Tumorigenic transformation induced by Ornithine and S-adenosylmethionine decarboxylases

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Search for common denominators in oncogenic signaling

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ABSTRACT

Cellular transformation, the transition of normal cells into morphologically and functionally altered neoplastic cells, may be caused by a great variety of genetic and epigenetic alterations and ensuing aberrations in cellular signaling. Here, we focused on studying the transforming mechanisms of fibroblasts, and searched for possible common denominators in oncogenic signaling.

The enzyme ornithine decarboxylase (ODC), which is the key regulator of the biosynthesis of polyamines, and essential for cell proliferation, has often been associated with neoplastic transformation. In normal cells ODC activity is strictly controlled, i.e. transiently increased in response to various growth stimuli, but becomes constitutively activated during cell transformation induced by carcinogens, viruses or oncogenes. The exact function of ODC in cell transformation has remained enigmatic. To test if ODC could be transforming by itself and potentially a common mediator of cell transformation, rodent fibroblasts were engineered to overexpress ODC in sense and antisense orientations. The constitutive overexpression of ODC induced full morphological transformation, increased cell proliferation and enabled anchorage-independent growth. The expression of ODC antisense or blocking the ODC activity with a specific inhibitor in cells transformed by v-*src*, in turn resulted in reversion of the transformed phenotype. These results indicate that ODC is both necessary and sufficient for cellular transformation of immortalized rodent fibroblasts and that oncogenes, such as v-*src* may, at least in part, exert their effects via ODC.

We compared signal transduction components induced by platelet derived growth factor (PDGF) in cells transformed by different oncogenes and *ODC*, for possible common points of convergence. *Src*, the first and best studied oncogene, and *Ras*, the oncogene frequently mutated in human cancers were used as representatives of broad-spectrum oncogenic transformation. By comparing v-*src* and c-Ha-*ras*^{Val12} transformed cells to ODC-induced cell transformation and to their normal counterparts we were able to exclude several signal transduction molecules, reported earlier to be activated in a transformation-specific manner. Instead, all transformed cell lines were found to display a constitutive increase in the phosphorylation of c-Jun on its transactivation domain.

Finally, the second key enzyme of polyamine biosynthesis, S-Adenosylmethionine decarboxylase was examined for its potential role in cell transformation. We showed that AdoMetDC overexpression surprisingly induces transformation both in sense and antisense conformations in rodent fibroblasts, and is highly tumorigenic in *nude* mice. AdoMetDC-induced transformation culminated in c-Jun phosphorylation on its transactivation domain, likewise to v-*src*-, *ras*- and ODC-transformed cells. Dominant negative mutants of MEK1 and JNK1 as well as the Jun mutant TAM67 (the transactivation domain deleted) reverted the transformed phenotype and TAM67 further effectively inhibited the anchorage-independent growth of the AdoMetDC-transformed cells.

AdoMetDC-transformed cells showed extremely aggressive growth in *nude* mice and the resulting tumors were characterized by chaotic neovascularization of a type of mosaicism in the tumor vessels. AdoMetDC-transformed cells were also very invasive *in vitro* and were shown to stimulate endothelial cell migration in 3D-matrigel assay. Angiogenic switch was triggered by an increase in VEGF expression and downregulation of thrombospondin-1 (TSP-1). The reintroduction of TSP-1 into AdoMetDC-transformed cells resulted in reduction of cell proliferation and ability to grow in soft agar. Extracellular matrix degradation and invasive capacity required for invasion and metastasis was associated with induction of matrix metalloproteinase-2 (MMP-2) and larger isoforms of Tenascin-C.

In conclusion, our studies on polyamine biosynthetic enzymes in cell transformation of rodent fibroblasts suggest an important role for ODC and AdoMetDC. Of specific interest, the AdoMetDC-transformed cells displayed very high invasiveness and chaotic angiogenesis in *nude* mice. Further, we identified several molecules potentially responsible for the angiogenic switch. By comparing the PDGF-induced signal transduction pathway of the ODC- and AdoMetDC-transformed cells to oncogenic Ras- and v-Src-transformed cells, we could identify c-Jun activation as a common point of convergence. Our findings are of interest not only for the field of polyamine research, but also generally for the dissection of relevant events in the multifaceted networks of signal transduction in various cell transformation models.

ABBREVIATIONS

AdoMetDC	S-adenosylmethionine decarboxylase
AKT	Ak strain transforming
AP	activator protein
ASK	apoptosis signal-regulating kinase
ATF	activating transcription factor
AZ	antizyme
AZIN	antizyme inhibitor
bZIP	basic leucine zipper
cAMP	cyclic adenosine monophosphate
CK	casein kinase
CM	conditioned media
CSC	cancer stem cells
CSK	cellular Src kinase
dcAdoMet	decarboxylated S-adenosylmethionine
DFMO	α-difluoromethylornithine
DN	dominant-negative
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF	eukaryotic translation initiation factor
EMT	epithelial-mesenchymal transition
ER	endothelial reticulum
FAK	focal adhesion kinase
GAP	GTPase activating protein
GDP	guanosine disphosphate
GDS	guanine nucleotide dissociation stimulator
GEF	guanine nucleotide exchange factor
GPCR	G-protein coupled receptors
GRB	growth-factor-receptor-bound protein
GSK	glycogen synthase kinase
GTP	guanosine trisphosphate
Ha-ras	Harvey rat sarcoma
HER2	human epidermal growth factor receptor
IRES	internal ribosome entry site
JAK	Janus tyrosine kinase
JH	JAK homology
Ki-ras	Kirsten rat sarcoma
LSF	late SV40 factor
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
MGBG	methylglyoxal bis(guanylhyldrazone)
MLK	mixed-lineage kinase
MMP	matrix metalloproteinase
MT-MMP	membrane-type matrix metalloproteinase
NCK	non-catalytic region of tyrosine kinase

NFκB	nuclear factor kappa B
N-ras	neuroblastoma rat sarcoma
ODC	ornithine decarboxylase
ORF	open reading frame
PAS	periodic acid Schiff stain
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PDK	3-phosphoinositide-dependent protein kinase
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIP	phosphatidylinositol phosphate
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PLP	pyridoxal phosphate
PTEN	phosphatase and tensin homolog
PTP	protein tyrosine phosphatase
PU	putrescine
RAL	Ras-related protein
RPTK	receptor protein tyrosine kinase
SAPK	stress-activated protein kinase
SEK	stress-activated protein kinase kinase
SFK	Src family kinase
SH	Src homology
SHC	Src homology 2 domain containing
SHP	SH2-containing phosphatase
SP	spermine
Sp1	specificity protein 1
SOS	Son of sevenless
SPD	spermidine
STAT	signal transducers and activators
TAK	HIV Tat-associated kinase
TERT	telomerase catalytic activity
TIMP	tissue inhibitor of metalloproteinases
TN	tenascin
TPA	12-O-tetradecanoylphorbol-13-acetate
TPL	tumor progression locus
TRE	tetracycline response element
TSP	thrombospondin
uORF	upstream open reading frame
UTR	untranslated region
VEGF	vascular endothelial growth factor

LIST OF ORIGINAL ARTICLES

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-IV.

I Auvinen, M, Paasinen, A., Andersson, L.C., and Hölttä, E.: **Ornithine decarboxylase activity is critical for cell transformation.** *Nature* 1992, **360**:355-358.

M. Auvinen has included this publication in her doctoral thesis, completed in 1997.

II Paasinen-Sohns, A. and Hölttä, E.: **Cells transformed by ODC, c-Ha-*ras* and v-*src* exhibit MAP kinase/Erk-independent constitutive phosphorylation of Sos, Raf and c-Jun activation domain, and reduced PDGF receptor expression.** *Oncogene*, 1997, **15:**1953-1966.

III Paasinen-Sohns, A., Kielosto, M., Kääriäinen, E., Eloranta, T., Laine, A., Jänne, O., Birrer, M., and Hölttä, E.: **c-Jun activation-dependent tumorigenic transformation induced paradoxically by overexpression or block of S-adenosylmethionine decarboxylase.** *J. Cell Biol.*, 2000, **151**:801-810.

IV Paasinen-Sohns, A., Kääriäinen, E., Yin, M., Järvinen, K., Nummela, P. and Hölttä, E.: **Chaotic neoangiogenesis induced by aggressive fibrosarcoma cells overexpressing S-Adenosylmethionine decarboxylase.** *Int. J. Biochem. Cell Biol.,* 2011, **43**:441-454.

TABLE OF CONTENT

INTRODUCTION

The polyamines putrescine, spermidine and spermine are involved in many fundamental processes of cell growth and survival, including association with nucleic acids, stabilization of chromatin structure, regulation of specific gene expression, differentiation, apoptosis, regulation of multiple ion-channels necessary for cell-to-cell communication and protection from oxidative damage and nucleic acid depurination [1-3]. Polyamines are indispensable for normal cell growth and their depletion results in cytostasis, a process by which cells stop dividing without dying [3, 4]. In contrast, when cells are stimulated to grow, polyamine synthesis is rapidly increased. The components of the polyamine biosynthetic pathway are highly conserved throughout evolution and the route harbors multiple tightly controlled steps in order to maintain a strict regulation of the polyamine levels [5, 6]. Even though dysregulated polyamine metabolism has frequently been associated with uncontrolled growth and cancer [7], evidence to indicate that polyamines have a contributing rather than an associative role in cancer has been lacking.

The first and rate-limiting step in polyamine biosynthesis, the production of putrescine, is catalyzed by ornithine decarboxylase (ODC). All tissues studied so far have demonstrated the requirement for ODC activity in cell proliferation. The ODC activity is strictly controlled in normal cells, but becomes transiently upregulated upon growth factor stimulation [8-10]. ODC activity is constitutive during cell transformation induced by carcinogens [11, 12], viruses [13, 14] or oncogenes [15-17].

The second important step in polyamine biosynthesis, the production of aminopropyl donor for the synthesis of higher polyamines spermidine and spermine is catalyzed by S-adenosylmethionine decarboxylase (AdoMetDC) [8, 18, 19]. Similar to ODC, it has a fast turnover rate and is rapidly induced in various normal and neoplastic growth processes [20, 21]. Inhibition of AdoMetDC by various drugs have been shown to have antiproliferative and antitumor activity and to inhibit metastasis [22-24]. However, direct evidence for the role of AdoMetDC in cell transformation has been missing.

Compelling data has indicated that the polyamine pathway is a downstream target for known oncogenes and that inhibition of polyamine synthesis disrupts the action of these genes [1]. This has led to revival in the interest of targeting polyamine metabolism as an anticancer strategy [3]. Understanding the molecular functions of polyamines and the role of their biosynthetic enzymes in cancerous growth would be essential in achieving these goals.

Transmission of the proliferative extracellular signals, like growth factor receptor activation by their ligands is mediated through the cytoplasm by cascades of activated cytoplasmic signaling molecules that ultimately lead to phosphorylation of transcription factors and to concomitant adaptation to the incoming signal [25]. Proliferation in normal cells is tightly regulated. Transformed cells, however, have escaped this control and can proliferate

independently from incoming signals [26]. One could reason that a transformed cell should display constitutive or improper activation of one or more components in the signaling pathways to gain independence of the growth regulation. Indeed, many of the molecules involved in various signal transduction pathways have been shown to be transforming in different cell lines. By comparing signal transduction pathways induced by the same activating growth factor, but in different oncogenic models, we searched for these possible points of convergence. These kinds of common relay points could have far-reaching importance, especially when designing new chemoprevention.

REVIEW OF THE LITERATURE

1. Polyamines and their biosynthetic enzymes

1.1 Properties of polyamines

Polyamines are polycationic molecules at physiological pH possessing a hydrocarbon backbone and multiple amino groups [18, 20]. Polyamines are present in all living organisms, with the most common polyamines being putrescine, spermidine and spermine [20]. The structures of these naturally occurring polyamines are shown in Figure 1.

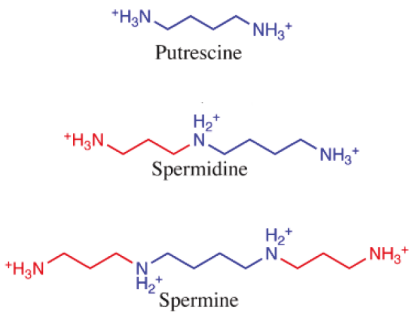

Figure 1: Putrescine, spermidine and spermine chemical structures

The positive charge on the polyamines is distributed along the entire length of the carbon chain. This distinguishes them from the point charges of the other cellular bivalent cations (e.g. Ca^{2+} or Mg^{2+}) and confers polyamines an ability to bind both specifically and non-specifically to numerous macromolecules, like DNAs, RNAs, membrane proteins, soluble proteins, and enzymes, and various small, negatively charged (eg. phosphorylated) molecules both in the cytoplasm and nucleus [27]. There is equilibrium between bound and free polyamines, the free polyamine pool representing 7-10% of the total cellular polyamine content. The concentration of free polyamines that are available for immediate cellular needs, is tightly controlled. This is vital for the cells, as a decrease in polyamine concentrations inhibits cell proliferation and excess is toxic [28, 29]. Polyamine concentration and composition do vary between species and tissues, and high concentrations are generally encountered in tissues having high turnover.

It has been amply studied and documented that polyamines are required for optimal growth in prokaryotes, eukaryotes, plants and animals [20, 30]. The vital importance of polyamines for normal cellular processes is further emphasized by their roles in maintaining chromatin structure, regulating ion-channels, maintaining membrane stability and scavenging free radicals [29, 31-34] (summarized in Figure 2). If polyamine metabolism is disturbed, a plethora of cellular processes may be affected, including structural changes in chromatin, DNA and RNA, transcription, translation, gene expression, autophagy and stress responsiveness as reviewed in [6]. Loss of polyamines consequently, has been shown to inhibit cell proliferation, differentiation, and regulate cell death [4, 35]. Confirmed by abundant research and corresponding data, the role of polyamines has been associated largely with cell growth and

cancer [8, 18, 20, 30], but more recently, also with aging [36-39], memory performance [40], neurodegenerative diseases [41] and metabolic diseases [42]. However, despite comprehensive studies addressing polyamines, and even after several decades since their discovery, a unifying concept to interpret the biochemical function of polyamines is missing. The direct and specific molecular functions of the polyamines can be still considered as one of the unresolved tasks of molecular cell biology [6].

However, a precise and essential function has been reported for spermidine: it is the substrate for the production of an unusual amino acid, hypusine [43], which is covalently bound in two-step process to a single protein reported to date, eukaryotic translation initiation factor 5A (eIF5A) [44]. Hypusinated eIF5A is required for efficient synthesis of proteins containing regions prone to stalling [45]. These are proteins regulating several key functions in growth and development, and thus, essential for the cell metabolism [3].

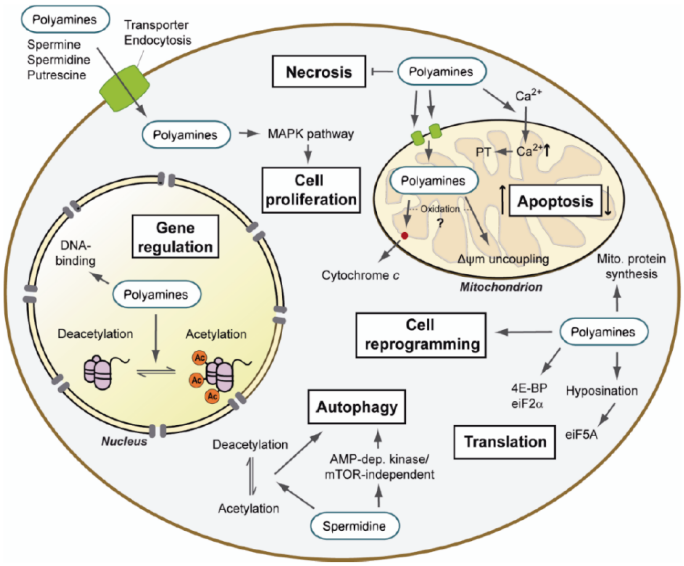

Fig. 2 Simplified outline of the cellular mechanisms induced by polyamines (from [39]).

1.2 Control of the intracellular polyamine content

The intracellular requirement for polyamines is maintained within a narrow range through the combination of a highly regulated metabolic pathway and transport systems. Regulation of polyamine levels is highly conserved throughout all known types of life, confirming its importance for the cell.

1.2.1 Polyamine biosynthesis

The major natural, biologically active polyamines identified in mammalian cells and tissues are derived predominantly from the amino acids ornithine and methionine. The established biosynthesis pathway (depicted in Figure 3) [8, 20] begins with ornithine decarboxylation, by ornithine decarboxylase (ODC), to form putrescine. Spermidine and spermine are formed from putrescine via addition of aminopropyl groups. These are donated by the methionine derivative decarboxylated S-adenosylmethionine (dcAdoMet) that is in turn produced by S-

adenosylmethionine decarboxylase (AdoMetDC). Spermidine and spermine synthases mediate the addition of aminopropyl groups and the latter two polyamines are catabolized by a single enzyme, spermidine/spermine acetyltransferase. In this reaction spermidine and spermine are acetylated creating putatively inactive acetylated forms, as acetylation reduces their positive charge, preventing their interaction with other molecules [6]. The acetylated forms can be converted back to lower polyamines or remain as acetylated polyamines, which are easily secreted from the cells. Further conversion to putrescine and spermidine, respectively, takes place via oxidizing reaction by polyamine oxidase. These reactions form a "polyamine back-conversion cascade" (reviewed in [29]) that allows the cell to regulate spermidine and spermine concentrations quickly, and thus, adapt their cellular activity or availability.

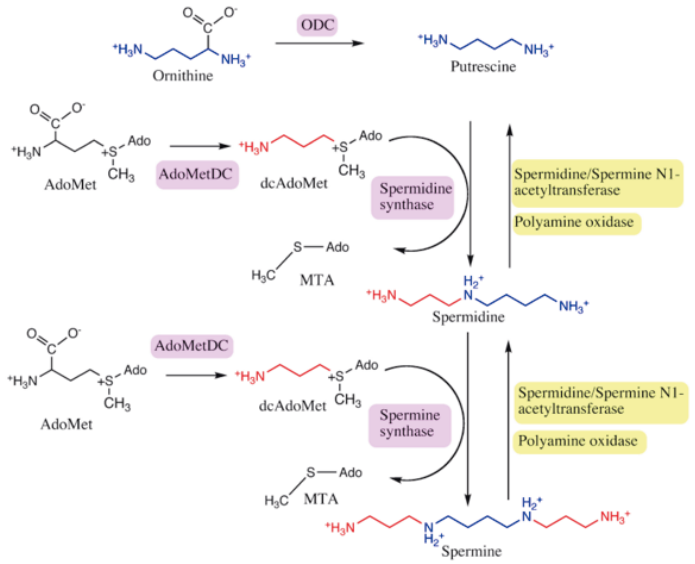

Fig. 3 Overview of the polyamine biosynthetic pathway (from [46]).

1.2.2 Polyamine transport

Polyamines play a crucial role in the regulation of cell growth and differentiation. Mammalian cells fulfill this requirement via the de novo biosynthesis of polyamines and/or through an efficient polyamine transport system obtaining polyamines from the extracellular environment. Polyamine transport has been detected in almost every model organism and are described both in bacteria and single cellular eukaryotes in molecular details [27]. In mammalian cells, however, polyamine-specific transport systems are less well understood.

Three models have been proposed (reviewed in [27, 47]), involving a different variant of the endocytic mechanism for the internalization step. After participation of a membrane permease polyamines can be processed through a series of endosomes or polyamines bind first to heparin sulfate moieties in glypican-1 at the cell membrane and are then internalized by endocytosis. It is also suggested that an uptake by caveolar endocytosis where polyamines are bound to a putative "polyamine receptor", could take place in gastrointestinal tissues.

The importance of polyamine uptake in malignant cells has been recognized for a long time. Transformed cells, in comparison to normal cells, accumulate exogenous polyamines at an increased rate [48]. In particular, highly activated polyamine uptake has been shown in several tumor cell lines [49]. Despite a lack of full understanding of the polyamine transport system, there has been considerable interest of employing it to deliver selectively cytotoxic agents into tumor cells [50].

1.3 Polyamine biosynthetic enzymes

The concentrations of rate-limiting enzymes in the polyamine synthesis and degradation pathways are regulated at different levels, including transcription, translation and protein degradation (reviewed in [51-53]). Polyamines can modulate the translation of most of the enzymes required for their own biosynthesis and catabolism through feedback mechanisms that are unique for each enzyme.

1.3.1 Ornithine decarboxylase

L-Ornithine decarboxylase (ODC) catalyzes the first and rate-limiting step in the polyamine biosynthetic pathway forming putrescine, which is then converted into higher polyamines spermidine and spermine (see Fig.3). Polyamine content plays important roles in both normal and neoplastic growth and alterations of polyamine synthesis via changes in ODC content occur in response to tumor promoters and carcinogens (reviewed in [52]). ODC can alter its activity in response to many types of cellular perturbations: it is induced in response to growth stimuli and in cells infected by viruses and under various pathological conditions such as cancer [8, 11-15, 30, 54, 55]. The loss of ODC gene in mouse embryonic development is shown to be lethal [56]. All this has guaranteed ODC constant attention not only as a prognostic factor, but also as a possible target for chemoprevention and therapy [7, 30, 57-59].

ODC is a pyridoxal phosphate (PLP)-dependent amino acid decarboxylase. For the ODC activity, the formation of a homodimer (51 kDa) with two active sites is required [60]. There are two domains in the ODC monomer, an NH_2-terminal domain that binds the cofactor, and a COOH-terminal domain. The active site is formed at the dimer interphase between these domains [61, 62]. The association between two ODC subunits is quite weak and the dimers are in rapid equilibrium with inactive monomers, even under normal physiological conditions [60].

1.3.1.1 Molecular characteristics of ODC

The regulation of ODC starts at transcription. The mammalian *Odc* promoter contains various elements (cAMP responsive element, CAAT and LSF motifs, AP-1 and AP-2 sites, GC-rich Sp1 binding sites, and a TATA box) that allow response to growth factors, hormones and tumor promoters [51]. Transcription factors that promote ODC transactivation include the oncogene c-*myc* [63] and NF B [64].

Like many other mRNAs that code for proteins that are important for cell viability and proliferation, ODC mRNAs have a long 5' untranslated region (UTR) which contains a strong secondary structure (reviewed in [52]). Translation is greatly enhanced by high levels of active eIF-4E, which is believed to be involved in the melting of secondary structures on mRNA 5'

UTRs [65]. The 5′ UTR contains two additional elements that reduce the efficiency of ODC mRNA translation: a small functional upstream open reading frame (uORF) and a GC-rich sequence [66-68]. uORFs are strongly inhibitory *in vitro* and *in vivo* to translation of ODC [68, 69], as the presence of them will prevent the ribosome machinery from initiating translation at the right codon in an efficient way. Mammalian ODC mRNA translation may also occur in a cap-independent manner using an internal ribosome entry site (IRES) [70] allowing translation to initiate even when cap-dependent translation is blocked, as in mitosis for instance [71, 72].

Polyamine concentration is a strong regulator of ODC translation (Fig. 4). An increase in intracellular polyamine levels leads to ODC translation repression, whereas a decrease causes translation activation. However, it is not well understood how this translation repression and activation by polyamines occurs [6]. RNA-interacting proteins that would act as translational repressors to ODC have not been reported.

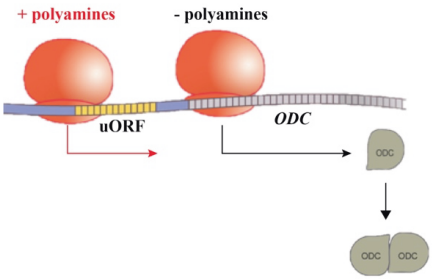

Figure 4 Polyamine concentration regulates ODC translation. In the presence of high polyamine concentration (+) ODC translation is repressed due to an uORF in ODC RNA. When polyamine levels decrease (-), ODC translation is activated, active ODC dimers can be formed and putrescine is synthetized (adapted from [6]).

1.3.1.2 Regulation of ODC activity

ODC has many unique features. Due to its very rapid turnover rate it is present only in minute amounts in normal growing cells [73]. ODC activity, which is very low in quiescent cells, increases upon exposure to trophic stimuli (incl. hormones, various drugs, tissue regeneration and serum growth factors) several folds within few hours [18, 74]. Even as stimulated, ODC encompasses only a small fraction of the total proteins in the cell. Addition of exogenous polyamines to the cells results in a profound and rapid fall in enzymatic activity of ODC [75].

1.3.1.2.1 Regulation by antizyme

The previously mentioned negative feedback abolishing the ODC enzymatic activity was shown to coincide with an appearance of another protein inhibiting its activity [76] depicted in Figure 5. This activity was called anti-enzyme for ODC, or antizyme (AZ) [77, 78]. ODC forms 1:1 complex with antizyme and its dissociation regenerates ODC activity [78]. The cell culture studies showed that there was a strong correlation between antizyme:ODC ratio with the degradation rate of ODC [77]. There are four members in the mammalian antizyme gene family, antizyme-1 being the best characterized. All members inhibit ODC activity, but are expressed differentially or in different abundancy [79].

Antizyme mRNA can be found widely, but in very low concentration in most mammalian tissues [76, 80]. However, in response to increase in polyamine levels, antizyme protein concentration rises rapidly through translational frameshifting [81]. Antizyme is encoded by two overlapping open reading frames (ORFs). The first (ORF1) is short, but it contains AUG codons that is able to initiate translation. The second (ORF2) encodes most of the protein, but

it needs the start codon from ORF1. This is possible by forward frameshifting one base before the stop codon of ORF1 is read. Translation continues in the new +1 frame to the end of ORF2 and produces active full-length protein products [82]. The frameshifting process is highly uncommon in most organisms; only three mammalian ODC antizyme genes use this translational control mechanism [83]. Neither the exact mechanism, nor how polyamines promote this frameshift have been clarified, yet.

In addition, antizyme can inhibit polyamine uptake and stimulate polyamine secretion [84, 85]. Again, exact mechanism for this remains unknown.

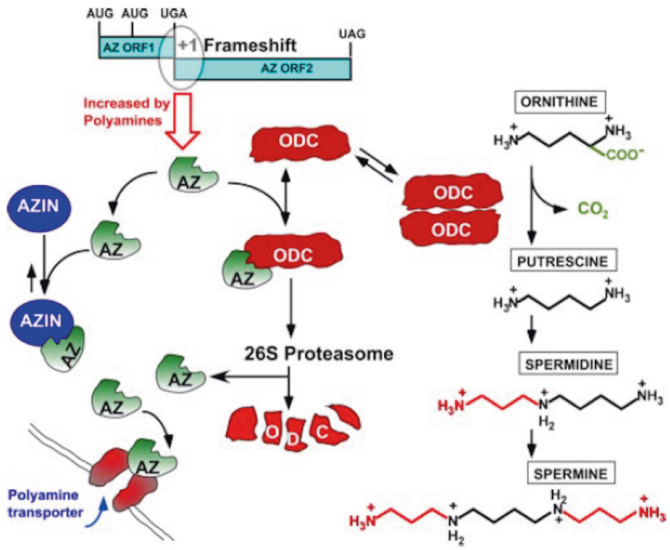

Figure 5. Functions of ODC, antizyme and antizyme inhibitor in polyamine metabolism. The ODC dimer catalyzes putrescine production, which is converted into spermidine and spermine. Antizyme (AZ) is synthesized via a frameshift in its mRNA translation, stimulated by polyamines. Antizyme interacts with ODC bringing it to proteasomal degradation, or binds to its inhibitor (AZIN) (from [51]).

Further, there are inhibitory proteins for antizymes. The first endogenous antizyme inhibitor (AZIN1) was first discovered as a protein that binds to antizyme and inhibits several of its functions [86]. Although AZIN as highly homologous to ODC was first thought to be a derivative of ODC, it was shown that AZIN is a distinct protein lacking the enzymatic activity of ODC [87, 88]. AZIN functions as a positive regulator of the polyamine pathway by binding to antizyme, which hinders formation of the antizyme-ODC complex and consequently suppresses antizyme-mediated ODC degradation [87, 89]. High AZIN concentration correlates with increased ODC protein levels and activity, enhanced polyamine synthesis and pronounced cell proliferation [89]. Interactions between ODC, AZ and AZIN are outlined in Fig. 5.

1.3.1.2.2 Degradation via the 26S proteasome

Similar to many short-lived proteins, ODC is degraded by the 26S proteasome [90]. Untypically, ODC is not first ubiquitinated, instead ODC antizyme binds to the COOH-end of the monomeric form of ODC, preventing thus its dimerization and presents the ODC to the 26S proteasome for degradation (Fig. 5) [77, 80, 82]. *In vivo*, in the absence of antizyme, ODC

is a substrate for the proteasome, with a half-life of 1-2 hours. When antizyme is present, the half-life of ODC is reduced to minutes [77].

In summary, ODC is delicately regulated not only at the levels of transcription and translation, but also by specific protein interactions controlling its enzymatic activity and degradation (summarized in Table 1).

Table 1. A short summary of ODC regulation

Responsive elements in *Odc* promoter	**Responsive to**	**Reference**
cAMP responsive element	Hormones	[91]
CAAT and LSF motifs	Hormones	[92]
AP-1 and AP-2 sites	Growth factors	[93]
GC-rich Sp1 binding sites	Multiple stimuli	[94]
TATA box	Multiple stimuli	[95]
TRE (TPA responsive element)	Tumor promoters	[96, 97]
Other regulatory target sequences in ODC gene	**Binds to**	
E-boxes (CACGTG)	Myc/Max transcription factor	[63, 98, 99]
ODC 3'-UTR, between bases 1851 and 2151	HuR (human antigen R), regulated by mTORC1	[100, 101]
Translational regulation of ODC synthesis	**Reported action on ODC mRNA**	
eIF-4E, long 5'-UTR in mRNA	Enhancement of translation by increased levels of eIF-4E	[52]
Short 5'-ORF and GC-rich sequence at the 5'-end	Strongly inhibitory to translation of ODC	[102]
Polyamines	Translation inhibition by excessive polyamines	[51]
Ras activation	Stimulatory effect on ODC mRNA content and translation	[103]
ODC protein regulated	**Reported action on ODC protein**	
Antizyme (AZ1, AZ2, AZ3, AZ4)	Inhibition and targeting ODC to degradation by complex formation	[77, 79, 80, 104]
Inhibitor of antizyme (AZIs 1-3)	Relieves ODC inhibition by binding to AZ	[79, 87]

1.3.1.2.3 ODC inhibition by DFMO

The recognition that polyamines and ODC activity are a prerequisite for cell growth and that polyamine biosynthetic pathway is frequently dysregulated in cancer resulted in the development of several inhibitors of this pathway [1, 18, 105]. α- difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor, is considered as the prototype inhibitor of ODC [106]. DFMO is a substrate for ODC and acts by binding to the active site of ODC, rendering the enzyme irreversibly inactive [105, 107]. Many studies using DFMO in tissue culture have shown near complete depletion of putrescine and spermidine, while effects on spermine concentrations differ [108]. This polyamine depletion results in the arrest of cell growth, reversible by the addition of exogenous putrescine [105]. Based on its cytostatic rather than cytotoxic effect *in vivo*, DFMO's success in cancer therapy showed, however, to be less effective than first expected [109, 110]. This could be a result of the poor access DFMO has into the cell and high doses that are needed to maintain the inhibitory effect. Also, the effect of different compensatory mechanisms, like upregulation of AdoMetDC and

increased uptake of circulating polyamines cannot be overlooked [1].

1.3.2 S-adenosylmethionine decarboxylase

S-adenosylmethionine decarboxylase (AdoMetDC) is the second rate-limiting enzyme in the biosynthesis of polyamines [111]. In various mammalian tissues the transition from a relatively quiescent state to rapidly proliferating cells is accompanied by large increases not only in the levels of ODC, but also AdoMetDC, and their biosynthetic products [112].

AdoMetDC catalyzes the formation of decarboxylated S-adenosylmethionine (dcAdoMet) from AdoMet that acts as a donor of aminopropyl groups for the synthesis of spermidine and spermine from putrescine. AdoMetDC, like ODC, is a member of a small group of enzymes that are dependent on a pyruvoyl group for the decarboxylation process [19, 21, 113]. AdoMetDC activity is highly regulated at multiple stages, including transcription, translation, proenzyme processing, catalytic activity, and degradation [29].

1.3.2.1 Regulation of AdoMetDC

1.3.2.1.1 Transcriptional regulation

Growth stimulation, e.g. through hormone treatment, tissue regeneration and cellular differentiation been shown to associate with increase in AdoMetDC levels [29, 114, 115]. Higher polyamines, spermidine and spermine negatively regulate the AdoMetDC gene transcription [8, 22, 116]. The exact mechanism, according to which AdoMetDC transcription is regulated in mammalian cells has not been fully clarified yet [6].

1.3.2.1.2 Translational regulation

AdoMetDC translation is mediated, as typical for polyamine pathway genes, by uORFs [117]. The mammalian uORF that precedes the ORF for AdoMetDC locates in very close vicinity downstream of the 5’ cap encoding a peptide: MAGDIS [118]. Upon synthesis of this peptide, ribosomes stall, blocking the entry to the AdoMetDC start codon. The length of the ribosome stall increases with raising spermidine and spermine concentrations [119].

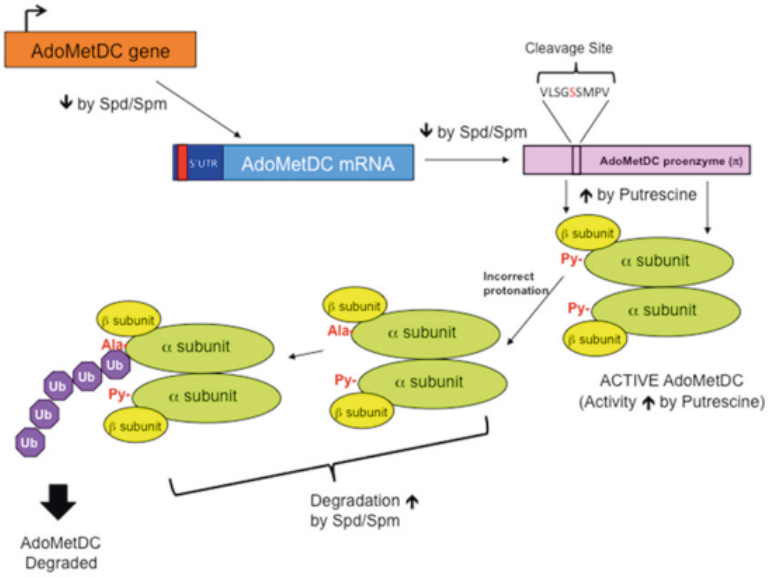

Figure 6. Regulation of AdoMetDC. Spd and Spm negatively regulate the gene transcription and mRNA translation, but increase the degradation of the enzyme. Putrescine activates the enzyme (from [29]).

1.3.2.1.3 Proenzyme activated by serinolysis

AdoMetDC is synthesized as an inactive proenzyme (Fig. 6). Through an internal serinolysis the proenzyme splits into two non-identical α and β subunits [120, 121]. Serinolysis generates a pyruvoyl group at the aminoterminus of the larger α subunit [122, 123] and AdoMetDC is rendered more prone for the decarboxylation reaction [46, 113].

The major regulators of AdoMetDC are polyamine levels. The diamine putrescine binds to the dimeric form of the enzyme [46] and affects the activity of mammalian AdoMetDC by accelerating the rates of processing of the inactive proenzyme to the mature enzyme [121, 124] and of decarboxylation by lowering the K_m for substrate AdoMet [22]. The roles of spermidine and spermine are more divergent, but at the end, their effect is more negative [111].

1.3.3 Half-life

The half-life of AdoMetDC has been shown to be usually 1-3 hours in most cell lines and tissues investigated [125], although a half-life of 3 minutes has been reported in a parasite [126] that is the shortest one found in eukaryotes. There is an inverse correlation between AdoMetDC half-life and cellular spermidine and spermine [29]: high polyamine levels accelerate degradation of AdoMetDC providing another control mechanism to maintain AdoMetDC activity and thus, polyamine levels under control [19].

1.3.3.1 Degradation via the 26S proteasome

Although both AdoMetDC and ODC activities are similarly regulated during cell proliferation, their route to the 26S proteasome degradation is different. It has been shown that AdoMetDC activity can vanish in a process known as substrate-mediated transamination [125]. In this reaction the amine group from the substrate AdoMet is transferred to pyruvoyl cofactor transforming it to an alanine [127], and leading not only to the permanent covalent inactivation of the enzyme, but also to a conformation change. This makes its ubiquitination site more accessible and thus more susceptible to degradation by ubiquitin-dependent 26S proteasome machinery [111, 125]. No interaction between AdoMetDC and antizyme has been detected [125].

1.3.3.2 Inhibition of AdoMetDC

As for ODC, the positive link between polyamine concentrations and cell growth has stimulated the development of several inhibitors targeting AdoMetDC [111, 128, 129]. The early experiments in mice and the clinical trial showing the antileukaemic effect of methylglyoxal bis(guanylhyldrazone) (MGBG) [130, 131] together with its ability to be a potent inhibitor of AdoMetDC and thus cell growth [105, 132] have led to development of other inhibitors based on the structure of MGBG [105, 133]. Especially one of them, SAM486A/CGP48664, was shown to inhibit AdoMetDC more specifically up to 90% and decrease the higher polyamine pools [1, 23]. However, besides the enzymatic inhibition, it stabilizes AdoMetDC against proteolytic degradation [134].

1.4 Polyamines and their biosynthetic enzymes in cancer

The association of polyamines to oncogenic and viral transformation and rapidly induced ODC activity upon exposure to growth-promoting factors and chemical carcinogens has been known for a long time [8, 30, 112]. Upon oncogenic transformation ODC activity is constitutively high ([15-17, 135]. ODC is also a target for c-MYC [63, 136] and the oncogenic activation of Ras induces ODC activity promoting malignant transformation and oncogenesis [15, 137]. When comparing cancer tissues to their normal counterparts, they were shown to have higher concentrations of polyamines, and the inhibitors of polyamine biosynthesis can inhibit tumor growth and metastasis significantly [138]. Activation of ODC resulting in increased concentrations of polyamines is associated with tumor promotion and progression [30, 139]. Furthermore, the expression levels of ODC correlate directly with the potential to promote tumorigenesis in both lymphomas and solid tumors [140, 141].

ODC has dominated the studies on the role of polyamine biosynthetic enzymes in cell growth and cancer and much less effort has focused on AdoMetDC. The loss of AdoMetDC gene, similarly to ODC, results in very early embryonic lethality in the mouse [142]. AdoMetDC is also induced by tumor promoters [141] and frequently dysregulated in cancer, with an important role in cell proliferation [1-3, 7].

2. Oncogenic transformation models

Protein phosphorylation has been shown to be important in virtually all cellular processes that control cell growth, proliferation and survival and are thus involved in development of the

transformed phenotype [143]. Kinases are a large family of enzymes, catalyzing the transfer of a phosphoryl group from a nucleoside triphosphate donor, such as ATP, to an acceptor molecule (reviewed in [144]). Tyrosine kinases are specifically catalyzing the phosphorylation of tyrosine residues and protein tyrosine phosphatases (PTPs) removal of it (reviewed in [145]).

The receptor protein tyrosine kinases (RPTK) are a varied family of transmembrane proteins that are able to bind to soluble and transmembrane ligands. As a result of ligand binding the catalytic activity of the receptor is stimulated leading to a coordinated cascade of signaling events that direct at the end a broad range of biological responses [146, 147]. RPTKs are able to recruit multiple auxiliary signal transducing proteins, e.g. Src, that act as downstream effectors [148].

2.1 Src

c-*src* was first isolated as the normal cellular homologue of v-*src*, the gene responsible for transformation of chick embryo cells by infection with Rous Sarcoma virus [149, 150]. It is the first proto-oncogene described and one of the first molecules demonstrated to have tyrosine kinase activity [150-154]. Src is a prototype model for studying cell transformation in general and understanding signal transduction involving tyrosine phosphorylation [150, 155]. In addition to Src, nine additional variants closely related to Src have been identified and are collectively termed Src family kinases (SFKs), Src, Fyn, and Yes being the more ubiquitous kinases [156, 157]. While Src is the most often implicated SFK in a range of human cancers [152], it was selected as one model system of transformation for your studies performed in murine fibroblasts.

2.1.1 Structure of Src

SFKs are defined by a common modular structure [158]: myristylation at the aminoterminus (required for the association with the plasma membrane) (M) [159], variable unique domain (U), SH3 and SH2 domains (SH = SRC homology), kinase domain (or catalytic domain), and a C-terminal tail containing a negative-regulatory tyrosine residue (R).

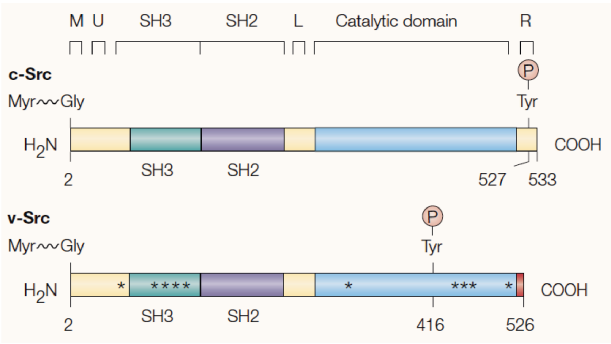

Figure 7. Structural comparison of v-Src and c-Src (adapted from [151]).

The kinase domain at the C-terminal half contains a conserved residue (Tyr 416) in an activation loop-region that is involved in autophosphorylation [160]. The autophosphorylation

at this position is required for full Src activation. Tyrosine 527 in Src provides one of the key regulatory attributes for Src and is a defining feature for all SFKs [161, 162]. v-Src, unlike Src, is constitutively active because of the lack of this decisive C-terminal negative-regulatory region (Y527 deleted) [163, 164]. As difference to c-*src*, v-*src* harbors additional point mutations throughout its coding sequence that have the potential to contribute to its high intrinsic activity and transforming capability [163]. The structure of v-Src and its cellular counterpart is illustrated in Fig. 7.

The SH-regions encode protein interaction domains: SH3 interacts with specific proline-rich sequences [165, 166] and SH2 domains bind to the negative-regulatory Tyr 527 (on kinase domain) [167, 168] and to the PDGF-R [169]. The "Src module" (comprising of an SH3, an SH2, and a kinase domain) is not only shared with other SFKs, but also with diverse and unrelated proteins involved in signal transduction [170, 171]. The concept that modular binding domains, like SH2 and SH3, govern the signal-triggered change in the localization of proteins, has now proved to be the most important consequence of the activation of tyrosine kinase pathways.

2.1.2 Activation of Src

Fig. 8 depicts, how the C-terminal tail, and both SH2 and SH3 domains contribute to the regulation of the Src kinase via intramolecular interactions [152, 155]. By binding back with its C-terminal phosphotyrosine to the SH2 domain, c-Src is inactivated. This and the additional interaction between the SH3 and the kinase domains form more closed structure in which the kinase domain cannot access its substrates. Src activation occurs when the C-terminal phosphotyrosine is removed enabling the opening of the Src molecular structure.

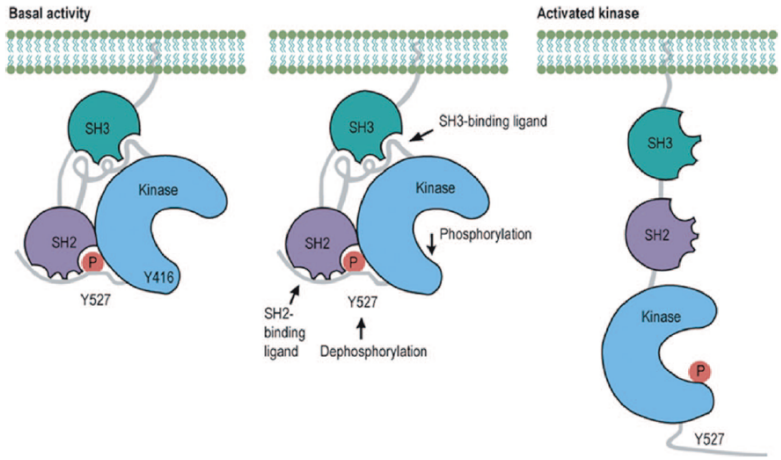

Figure 8. Activation of c-Src (from [157]).

Phosphorylation of C-terminal phosphotyrosine is carried out by another kinase, the Cellular Src Kinase, CSK [172], and for the dephosphorylation the function of various phosphatases like Protein Tyrosine Phosphatases (PTPs) and SH2-containing phosphatases (SHPs) have been suggested [173-175]. The opening of the molecule frees the SH2 and SH3 domains to interact with numerous cellular factors, including receptor tyrosine kinases [176, 177], G-protein-

coupled receptors [178], and focal adhesion kinase (FAK) [179], (depicted in Fig. 9), among many others [180]. The activated Src is a protein of multiple functions and is involved in the regulation of normal and oncogenic processes, like proliferation, differentiation, survival, motility, and angiogenesis [152, 177].

2.1.3 Src localization

The correct localization of Src with the plasma membrane is considered to be requisite for its transforming activity [181] (Fig. 9). The autophosphorylation of Tyr419, which occurs when Src is targeted to the membrane enabling the interaction with activated receptor tyrosine kinases, enhances Src transforming activity to the highest level [182].

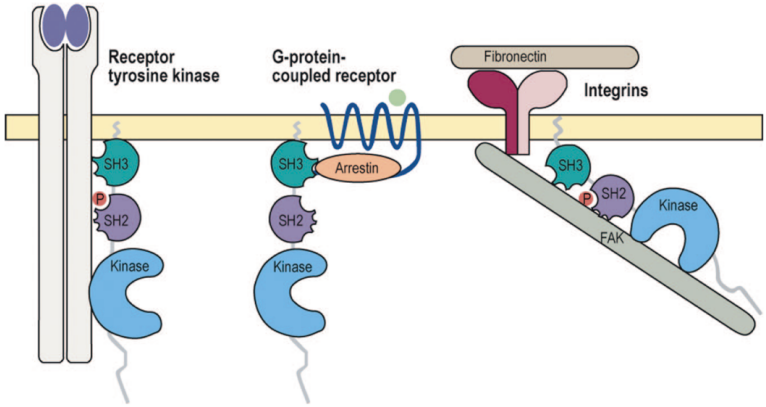

Figure 9. Simplified view of c-Src regulation by plasma membrane receptors (from [157]).

2.1.4 Interaction with substrates

Src applies its effects on tumor-cell behavior through interactions with various substrates and binding partners. These initiate various signal transduction cascades that are illustrated simplified in Figure 10. Research done on Src has delivered abundant data on signal transduction in general, however, the exact function of Src itself within a cell has remained ambiguous.

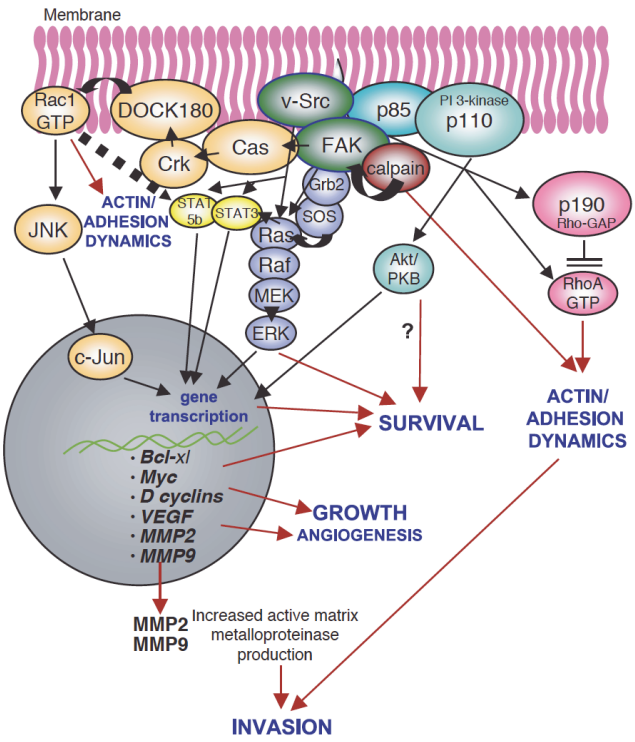

Figure 10. Selected signal transduction pathways involving v-Src (from [183]).

2.2 Ras

The Ras genes represent some of the earliest described oncogenes, and they and the signal transduction pathways induced by them have fundamentally transformed the present understanding of cancer biology. Originally identified as a viral component able to induce sarcomas in rats [184, 185], and later found to be normal components of the human genome [186, 187], Ras genes were shown to transform normal animal [188] and human cells [189, 190]. Now it is known that Ras alterations contribute to 20-30% of all human cancers and the Ras signaling pathway has been identified as the most altered oncogenic network in cancer [191]. Ras is the prototype of a larger family of proteins (~150) that are small GTPases indicated in regulation of cell growth, proliferation and differentiation [192]. Small GTPases are enzymes catalyzing the hydrolysis of guanosine triphosphate (GTP) to guanosine disphosphate (GDP) [193]. Ras GTPases cycle between the active GTP-bound state and the inactive GDP-bound state; they are inactivated by GTPase activating proteins (GAPs) and activated by guanine nucleotide exchange factors (GEFs) [194]. Activated GTP-bound Ras binds to and activates in turn a variety of downstream effectors.

2.2.1 The Ras family

The Ras gene family consists of three isoforms – Ha- (Harvey), N- (neuroblastoma) and Ki- (Kirsten rat sarcoma viral oncogene homolog) *ras*. Expression of these genes is very abundant and conserved across various species, emphasizing their important roles in many normal

cellular processes, including proliferation, differentiation, and cell death [195]. These isoforms are highly homologous regarding their primary amino acid sequence (~80%) and differ only in the C-terminal hypervariable domain [196]. This domain contains sequences that are necessary for the Ras proteins to anchor on the cytoplasmic side of the cell membrane [197], where each isoform differentially regulates its downstream effectors, resulting in distinct alterations both in the strength and the type of signal that is produced [196, 198-200].

2.2.2 Regulation of Ras

Oncogenic activating mutations in all three Ras genes are found in 27% of all human cancers and have been shown to concentrate at positions 12, 13 and 61 in all of the isoforms (COSMIC v90) [201]. The GAP-mediated GTP hydrolysis is defective in mutant Ras resulting in an accumulation of constitutively GTP-bound (active) Ras in cells. As the consequence of the persistent active Ras, the downstream effector pathways are constantly activated. The mutated isoforms can be found in human cancers with different frequencies: K-Ras predominates with 85%, followed by 11% of mutated N-Ras and the least mutated H-Ras with 4%. There is evidence that these mutations do not have only differential oncogenic potencies, but also distinct functional consequences [200, 202, 203], adding even more complexity to the signaling networks controlled by Ras [201].

The normal function of Ras proteins requires them to be post-translationally modified in order to localize correctly to their subcellular compartment on the plasma membrane. This attachment of Ras proteins to cellular membranes is essential for them to be biologically fully functional. The newly synthesized Ras isoforms in cytoplasm undergo several posttranslational modifications, like farnesylation, palmitoylation and/or ubiquitination [195, 204]. These modifications control Ras proteins' traffic via the ER-Golgi secretory pathway to their specific final destinations on the inner side of the plasma membrane [205]. At the plasma membrane different Ras isoforms can laterally segregate into spatially distinct and dynamic nanoclusters that are the sites for specific effector recruitment and activation, leading further to the signal propagation [206]. The membrane attached Ras cycles between inactive GDP-bound and active GTP-bound stages. Several GEFs (guanidine exchange factors) and a variety of GAPs (GTPase activating proteins, which switch off RAS by catalyzing RAS-mediated GTP hydrolysis) have been distinguished for Ras [207-209]. Finally, it is the balance between the GEFs and GAPs that determines the activation state of Ras and its downstream effector pathways [210].

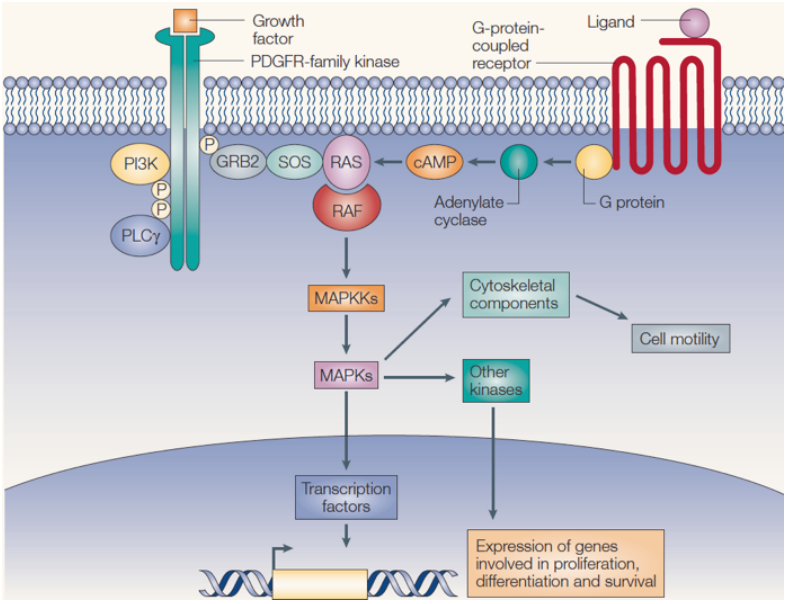

Figure 11. Schematic view of Ras activation and signaling taking place at the plasma membrane (from [211]).

Figure 11 illustrates, how the activated receptor tyrosine kinase (in this case the PDGF receptor) [212, 213] upon ligand binding is autophosphorylated, creating docking sites for several SH2-domain containing adapter proteins, such as Grb2 (growth factor bound protein 2) [214] or SHC [215, 216] bind to the activated receptor. Grb2 in turn binds to Sos (an example of several Ras-GEFs) [207, 209] via its SH3-domains and couples the whole complex to the plasma membrane, where Ras is also localized. Due to the close vicinity of Sos and Ras, the GTPase activity of Ras is increased, resulting in replacement of GDP by GTP and thus, Ras activation [195].

2.2.3 Ras-induced signal transduction pathways

Ras signaling can be activated not only by a number of cellular receptors including receptor tyrosine kinases (RTKs) as depicted above, but also by G-protein coupled receptors (GPCRs) and integrin family members (see Fig. 11). Commonly, these receptors initiate Ras signaling cascades through assembly of several scaffolding proteins that have the main task of mediating conversion of Ras from an inactive GDP-bound form to an active GTP-bound state [217]. This induces a conformational shift in the Ras protein enabling it to interact physically with several alternative downstream signaling partners.

The spectrum of possible Ras effectors is broad, but the four main pathways that are activated downstream of Ras and previously shown to be relevant for cellular transformation are illustrated in Fig. 12: Ras-Raf-MAPK, PI3 kinase, RAL–GDS and phospholipase C (PLC) pathways. They have major roles in mediating signals that control cell proliferation, survival, adhesion, and motility [192, 195, 203, 218].

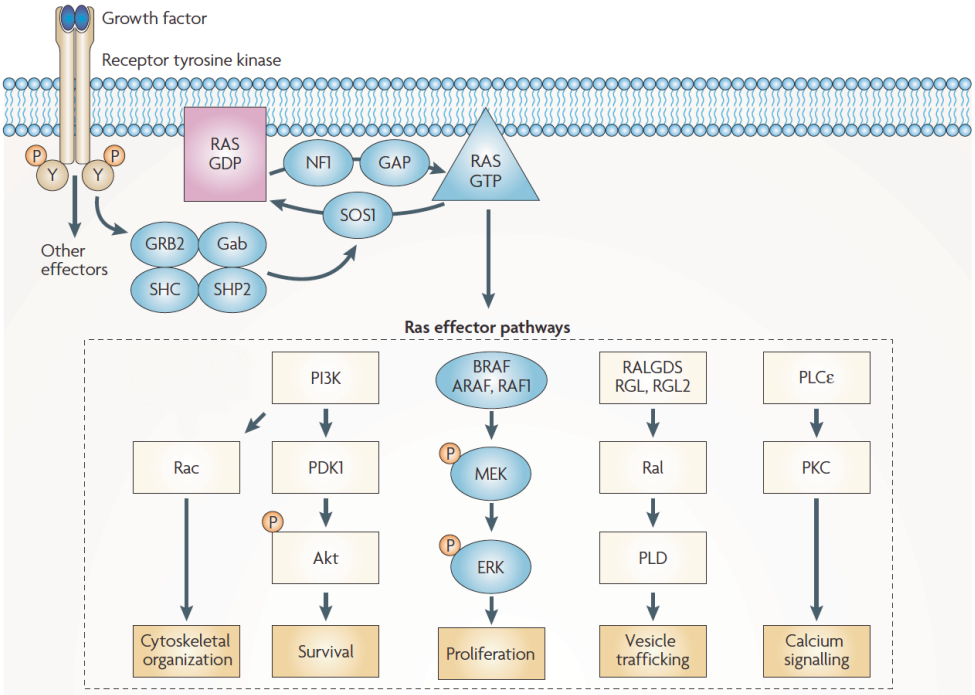

Figure 12. Ras-induced signal transduction pathways (adapted from [219]).

2.2.3.1 Ras-Raf-MAP kinase pathway

From the various Ras effectors, the first one to be characterized was the protein serine/threonine kinase Raf. The activated Ras binds to, and contributes to the activation of, the three closely related Raf proteins (c-Raf1, Braf and Araf). The binding of Ras triggers Raf's relocation to the plasma membrane, which is required for its activation [220, 221]. Downstream of this, activated Raf phosphorylates and activates a second mitogen-activated protein kinase known as MEK – a dual-specificity kinase that is capable of phosphorylating both serine/threonine and tyrosine residues, and activating thus the mitogen-activated protein kinases (MAPKs) Erk1 and Erk2 (extracellular signal-regulated kinases 1 and 2). Once activated, Erk kinases not only phosphorylate cytoplasmic substrates, but can also translocate to the nucleus, where they cause the phosphorylation of transcription factors, like AP-1 [222] and MYC [223], and lead to the initiation of the immediate and delayed early gene responses. Consequently, central cell-cycle regulatory proteins (e.g. D-type cyclins) are expressed, enabling cells to proceed through the cell cycle [224]. Hence, Raf activation promotes cell-cycle progression, at least in contribution of other signals [225]. The Ras-Raf-MAPK pathway is reviewed in [150, 195, 226].

2.2.3.2 PI3 kinase (PI3K) pathway

The phosphatidylinositol-3-OH kinases are a large family consisting of several classes and isoforms [227, 228]. The classification is based on their variable specificity for substrates and differences in structure. The common domain structure for all classes is heterodimer between

a p110 catalytic and a p85-type regulatory subunit. Upon activation of RTK a p85-subunit attaches to phospho-tyrosine residues and/or other adaptors found on the RTKs. As a result, the catalytic subunit p110 is disinhibited and phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). The direct interaction of Ras with the catalytic subunit (p110) of PI3Ks that has translocated to the plasma membrane and went through conformational changes, can also lead to activation of this lipid kinase [229]. Conversely, the tumor suppressor protein PTEN dephosphorylates PIP3 back to PIP2, resulting in termination of signaling [230]. By binding of PIP3 to a large number of proteins through the PH (pleckstrin homology) and other domains, PI3K controls the activity of a plethora of downstream enzymes. Once PIP3 is formed, it recruits PDK1 (3-phosphoinositide-dependent protein kinase-1) and AKT (or protein kinase B, PKB) kinases and brings them in close proximity, where PDK1 can phosphorylate AKT [231-233]. Phosphorylation of many targets by AKT is strongly anti-apoptotic [234] and, hence, an important part of the survival signal that is generated by Ras [235].

2.2.3.3 Ral-GDS pathway

Ras-related (Ral) proteins belong to the subfamily within the Ras family of small GTPases and through one of its exchange factors Ral-GDS (Ral guanine nucleotide dissociation stimulator), Ras is able to stimulate Ral [236, 237]. Activation of Ral-GDS pathway has been implicated in activation of phospholipase D1 and together, interacting with PI3K pathway to contribute to cell cycle arrest, transcription and vesicle transport [195]. Noteworthy, the activated Ral-GDS pathway appears to have more critical function in Ras-induced oncogenesis in human cell lines *in vitro* [238, 239], indicating the difference to murine Ras-transformation models in which the Raf-MAPK pathway has the dominant role. Recently shown efficacy of the Raf-MAPK pathway inhibition in various KRAS-mutated human tumor cell lines, however, emphasizes the emerging importance of this pathway also in the human cells [223, 240].

2.2.3.4 PLC pathway

Phosphoinositide-specific phospholipase C proteins (PLCs) are a family of enzymes that regulate the hydrolysis of the inner plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) [241]. As a result, two intracellular second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) are produced, and protein kinase C (PKC) is activated and internally stored Ca^{2+} released, respectively.

Initially, the activation of phospholipase C and the inositol lipid signaling pathways was thought to occur primarily by activating heteromeric G proteins or tyrosine kinases [242, 243]. Upon mitogen stimulation, like platelet-derived growth factor (PDGF), the autophosphorylation of the receptor creates a binding site for the PLCγ-isozyme that contains SH2- and SH3-domains capable to specifically interact with tyrosine residues [244, 245]. Even though the PDGF receptor activation was shown to induce concomitant and rapid increase in GTP-bound active Ras, data about the role of Ras in PLCγ activation has been contradictory [213, 246, 247]. Later, the expansion of the PLC-isozyme family with an isotype epsilon (PLCε) has brought clarification. PLCε binds to, and is regulated by, activated Ras [248, 249]. The PLCε that contains RA (Ras-association) domains instead of SH-domains, was found to bind Ras in a GTP-dependent manner, comparable to the binding of Ras to Raf-1 [248]. Meanwhile, eleven mammalian PLC isoforms have been identified with structural differences, which may be

linked to the distinct mode of regulation of each isoform [250], creating even more diversity to this complex pathway (reviewd in [217]).

2.2.4 Aberrant Ras-mediated signaling in transformation

As described above and depicted in Fig 12, Ras oncoprotein has a plethora of effectors and it is able to activate multiple downstream signaling pathways. The role of oncogenic Ras in the activation of Raf-MAPK signaling pathway both in experimental models and human malignancies is richly documented. More than a century after its finding [251] and an excess of publications since then have shown that GTP-bound RAS is able to bind and turn on effector enzymes activating pathways that control cell proliferation, survival and other aspects of cell behavior contributing to the transformed phenotype [196, 205, 217, 231, 252]. The Ras pathway has been shown to be one of the most prevalent oncogenic alterations in both experimentally induced animal and human tumors [195, 200, 201, 203, 204, 227, 231].

An important concern perplexing the view of Ras transformation is that the different Ras-induced pathways have been reported to be mechanistically distinct depending on the species and/or cell type. As the Raf-MAPK pathway has been shown to be solely responsible of transformation of immortalized mouse fibroblasts [253, 254], its activation is not enough to transform intestinal epithelial cells originating from rat [255], human MCF-10A breast cancer cell lines [256], or human BJ fibroblasts [238]. However, the identification of mutated BRAF leading to the constitutive activation of Raf-MAPK-pathway in melanomas and in lesser extend in other human tumors ratifies the importance of this Ras- effector pathway in human malignancies [257]. There are also differences in requirements for Ras-induced transformation among different cells even of the same species: in human HEK cells concurrent activation of PI3K- and Ral-GDS pathways transforms cells, while in human fibroblasts Raf-MAPK and Ral-GDS pathways need to be perturbated for full transformation [258].

Even though research on oncogenic Ras has provided rich and instructive information about structure, biochemistry and biology of Ras, many issues are still poorly understood and much remains to be elucidated. The structure of the Ras isoforms is very similar, but they appear to have distinct effector pathways, which together with their specific and differential membrane location or subcellular compartmentalization [217, 259, 260] and in certain cell, tissue or species specific context, may even have opposite responses or very different preponderance [201, 203, 205, 261-263]. Also, the expression levels of oncogenic Ras impact the degree of aberration in signaling (see publication II, [264]) and perhaps through this, phenotypic outcome. There is an increasing number and complexity between interactions of the Ras effectors and the downstream pathways they induce, including feedback loops and signaling redundancy [265-267], making understanding of Ras-signaling network in different context even more challenging.

2.3 Cell transformation in rodent and human cells

Transformation of a normal cell into a cancerous cell is considered to occur by accumulation of genetic errors in a multi-step process [268]. The evidence is now substantial that there are differences in rodent and human cell transformation; the type of signaling pathway disturbed and how many aberrations are required [269, 270]. This seems to vary according to the cell type and tissue and even among the same organism. One of the key reasons for the differential

resistance to oncogenic transformation between murine and human cells could be due to differences in DNA repair capacity, exerted by expression of TERT (telomerase catalytic subunit) [271, 272]. Rangarajan et al [258] have determined that for transformation of normal mouse fibroblasts only two signaling pathways need to be perturbated (involving Raf-MAPK pathway and p53), instead of six pathways in human fibroblasts. Indeed, several sets of genes have been identified to cooperate to transform human fibroblasts, commonly including one of the Ras-pathways, c-Myc and telomerase activation, the perturbation of protein phosphatase 2A and the inactivation of tumor suppressors, like p53, Rb and PTEN [258, 273-275]. In this respect there are still unanswered questions, as these gene sets appear not to be universal to all human cell lines: some human fibroblasts continue to be refractory to transformation even under these conditions [276], suggesting the existence of still unidentified factors required for cell transformation specifically in human cells. Consequently, there are limitations of rodent experimental systems that are important to keep in mind for attempts to apply murine studies of *RAS* in human malignancies [192, 269, 270].

3. Signal transduction

3.1 Function of protein kinases and phosphatases

Protein phosphorylation and dephosphorylation, catalyzed by kinases and phosphatases, can modulate the function of a protein in various ways; its biological activity is increased or decreased, it can be stabilized or marked for destruction, it can change its subcellular localization or assembly of signaling complexes is disrupted [277]. The simple, sensitive and dynamic way of regulation of proteins by phosphorylation on serine, threonine and tyrosine residues have made it the most general control mechanism in eukaryotic cells, able to influence pathways leading from cell division to cell death [278]. The most of the phosphorylation events occur on serine and threonine residues, whereas tyrosine phosphorylation is considerably rarer post-translational modification [279]. The discovery of protein kinases associated with viral transforming proteins and the capacity to phosphorylate tyrosine residues suggested that tyrosine phosphorylation plays a significant role in growth control [153, 280]. Aberrant phosphorylation is acknowledged now as a cause or consequence of many human diseases, and oncogenic mutations resulting in RTK overexpression and/or constitutive activation have been shown to be associated with many types of cancers [277, 281].

Much less attention has been paid to the counter players of the balance, namely protein phosphatases [282-284]. Protein tyrosine phosphatases (PTPs) comprise a large and structurally diverse family that is divided in receptor-like or non-receptor-like forms [285]. The receptor-like PTPs traverse plasma membrane and have extracellular domains resembling those of cellular adhesion molecules with immunoglobulin-like domains and fibronectin-III-like repeats and cytoplasmic part with PTP domains containing catalytic domain responsible for dephosphorylating activity. In non-receptor PTPs the catalytic domain is often linked to domains that mediate protein-protein interactions, like SH2-domains in SHP2 [286], connecting it physically to e.g. the most RTKs like PDGF receptors (see later).

As PTPs can antagonize RTK signaling by direct receptor dephosphorylation, they have been almost naturally considered as potential tumor suppressors and indeed, several inactivating mutations or other tumor suppressive evidence have been identified in human cancers or in

vivo models [287, 288]. The PTPs can exhibit also oncogenic activity, exemplified by SHP2 that is required for the activation of MAPK pathway upon growth factor stimulation and is considered as a proto-oncogene [282, 286, 289].

Like tyrosine phosphorylated proteins the serine/threonine phosphorylated proteins have specific phosphatases (PSPs) that are reversing the actions of serine/threonine kinases [290]. One of the three major PSP-families is the family of phosphoprotein phosphatases (PPP), in which the catalytic subunit associates with a large number of regulatory subunits enhancing the coverage of broader spectrum of targets. One important representative of the PPP-family is PP2A whose several isoforms have been shown to function mainly as tumor suppressors [291], but also tumor promoting roles, depending on the context of the cancer cell, have been reported [292, 293]. Interestingly, CIP2A, the cancerous inhibitor of PP2A that has been observed overexpressed or constitutively activated in various human cancers [294, 295], has proven to be an attractive target for designing new cancer therapies (summarized in [296]).

3.2 PDGF-signaling pathway

The platelet-derived growth factor (PDGF) receptor is one of the best-characterized growth factor receptors [150, 212, 297, 298]. PDGF isoforms stimulate growth, survival and motility of mesenchymal cells and by activating their receptors, mediate central signals during the embryonal development and control tissue homeostasis in the adults [299, 300]. The overactivity of PDGF receptors has been found to drive tumor cell growth [301]. As several signal-transducing proteins that are thought to be involved in PDGF-induced signaling are well characterized and as both Src family kinases and Ras proteins are involved in PDGF-induced signaling in normal cells, it was our choice of research model to study this signal transduction pathway in transformed cells.

3.2.1 PDGF isoforms

The PDGF family of growth factors is composed of four different polypeptide chains encoded by four different genes [301, 302]. The classical PDGF chains, PDGF-A and PDGF-B, undergo intracellular activation during transport in the exocytic pathway and more novel PDGF-C and PDGF-D chains require activation by extracellular proteases [212, 302]. The four PDGF chains assemble into dimers via homo- or heterodimerization (Fig. 13, upper part), and five different dimeric isoforms have been described: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. The later discovered PDGF isoforms (CC and DD) will not be discussed, as they have not been relevant for the work presented here.

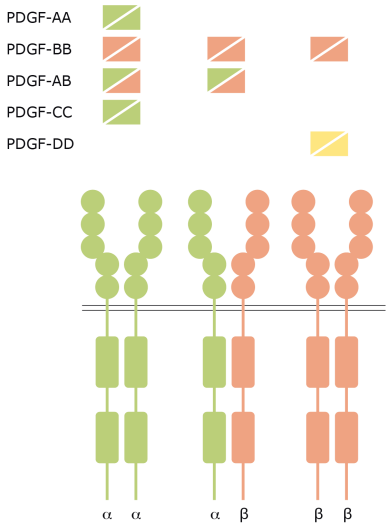

Figure 13. The active PDGF receptor complex consists of two chains associated with one dimeric ligand (redrawn from [212]).

3.2.2 PDGF receptors

PDGFR is the member of the type III class of RTK receptors that are typically formed by five Ig-like domains extracellularly and a split kinase domain intracellularly [212]. Ligand binding results both in homo- and in heterodimerization of PDGF-α and -β receptors (Fig. 13, lower part). The dimerization is a central step in receptor activation since it brings the intracellular parts of the receptors close to each other promoting autophosphorylation of certain tyrosine residues in the intracellular parts of the receptors. The PDGF polypeptide chains bind to the receptors with different affinities and different dimeric receptor complexes having overlapping, but slightly dissimilar signaling abilities. The receptors have several autophosphorylation sites (Fig. 14) that serve critical functions: the conformation changes of the intracellular parts of the receptors promote receptor activation, and provide docking sites for SH2-domain-containing signal transduction molecules [212, 298].

3.2.3 PDGF receptor induced signaling

Several SH2-domain-containing molecules selectively bind to autophosphorylated residues in the PDGF receptors and become activated by them (reviewed in [212, 298, 299] and depicted in Fig. 14): tyrosine kinases of the Src family [303], the SHP-2 tyrosine phosphatase (or Syp) [304, 305], phospholipase C-γ (PLC-γ) [244] and the Ras-GAP [306] are molecules with intrinsic enzymatic activities. The receptors bind directly also STATs (signal transducers and activators of transcription) that translocate after their activation into the nucleus to function there as transcription factors [307-309]. Adaptor molecules without intrinsic enzymatic activities, like the regulatory subunit p85 of the phosphatidylinositol 3' -kinase (PI3K) [310], which forms complex with the p110 catalytic subunit, and Grb2 [311] which binds and phosphorylates the nucleotide exchange molecule SOS1 [312] have determined docking sites on the intracellular part of the receptor as well. Additionally, PDGF receptors bind to and phosphorylate other

adapter molecules, e.g. Shc [313], and Nck [314, 315], which in turn promote further interactions with various different downstream signaling molecules.

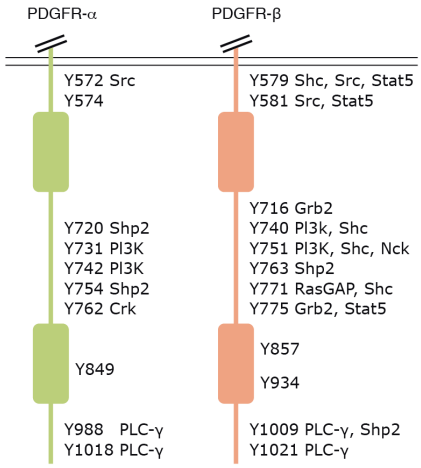

Figure 14. PDGF receptors present several possible sites for autophosphorylation (PDGFR-α 10 and PDGFR-β 11) that can bind SH2-containing signaling molecules (redrawn from [212]).

The phosphorylation of PDGF receptors at multiple sites causes the binding of various signaling molecules that form complexes with other molecules leading finally to the activation of signaling pathways illustrated in Fig. 15. These pathways conduct the signal from the plasma membrane to the nucleus, where its effect is exerted on cell proliferation and survival, as well as on actin reorganization and cell migration.

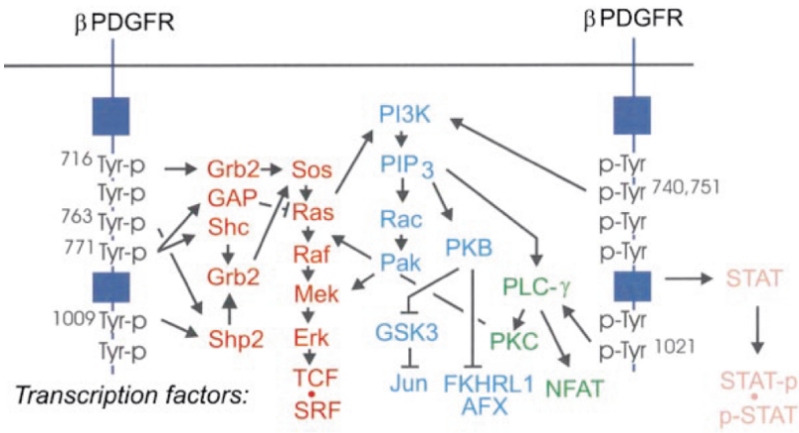

Figure 15. Signaling network from the activated PDGFRβ consists of multiple interconnected pathways from receptors to the nucleus (from [25]).

3.3 JAK/STAT pathway

Janus tyrosine kinase (JAK) family of proteins and signal transducers and activators of transcription (STATs) comprise a signaling pathway that has been shown to exert a role in development, proliferation, differentiation, and survival of cancer cell [308, 309]. Compared to other cell signaling pathways, JAK/STAT pathway seems with relatively few components, simple and straightforward; the mammalian JAK family contains four, and STAT family seven members that act mainly as transcription factors [316, 317]. It is a highly conserved pathway and observed in a wide range of species, activated primarily by cytokines [318], but also by growth factors [307]. JAKs belong to the family of non-receptor PTKs and lack SH2 or SH3 domains, but contain five upstream repeats referred to as JAK homology (JH) domains and two PTK-like domains in the C-terminal half of the protein. These two almost identical phosphate-transferring domains have given the non-receptor kinases the name “Janus”: one domain exhibits the kinase activity, while the other negatively regulates the kinase activity of the first [319]. The supposed relative simplicity of the pathway has been found over the years to be more complex due the inter-pathway cross-talks, like the effect of STAT3 activation on Ras and PI3K/Akt pathways [320], and the connections of JAK2 to PI3K and ERK pathways [321].

Treatment of cells with PDGF and EGF has been shown to specifically induce STAT1 (p91/p84 or p91) and STAT3, which become phosphorylated on tyrosine residues by JAKs and translocate into the nucleus where they act as transcription factors [307, 316, 317, 322]. However, details about these interactions have remained limited.

The role and significance of dysregulated STAT signaling in cancer biology have been studied intensively. According to major evidence STAT1 activation has rather a suppressive function in cancer cells [323]. Yet, some experiments and clinical studies suggest that STAT1 also exerts tumor promoter effects under specific conditions, and dependent on the cellular context, STAT1 can have either oncogenic or tumor suppressive function in the same cell type [324].

Several human cancers present constitutive overexpression or tyrosine phosphorylation of STAT3 [325] that supports transformation of various cell culture and animal models [326]. Constitutively active mutants of Stat3 have been shown to be able to transform normal cells into cancer cells [327].

3.4 S6 kinase pathway

The interest towards the possible role of S6K in transformation was evoked by the finding that Ras-transformed cells showed increased ribosomal protein S6 phosphorylation [328]. As part of the 40S ribosomal subunit it is supposed to be involved in regulating translation and mouse model studies show its involvement in the regulation of cell proliferation [329]. The enzymes p70-S6K1 and p85-S6K1 (two isoforms of the same enzyme) that are activated by a complex phosphorylation event [330] are responsible for the S6 phosphorylation, however, independent of Ras and MAPK pathway [331].

3.5 p38 MAP kinase pathway

p38 MAP kinase is a member of MAP kinase family that is activated in response to a variety of extracellular stimuli including stress, cytokines and growth factors (like PDGF) [332, 333]. It has been shown to phosphorylate several transcription factors, e.g. ATF-2, STAT-1 or the Max/Myc complex [333] and indirectly regulate activating protein-1 (AP-1) activity [334]. Although one of the major functions of the p38 kinase pathway is regulation of inflammation and apoptosis, in many cases the biological consequences of p38 kinase activation overlap with those of ERKs in mediation of cell growth and differentiation [335, 336]. p38 MAP kinase has been linked to the normal and oncogenic activity of Ras [337].

3.6 JNK pathway

Jun N-terminal kinases or JNKs are involved in a wide array of signaling events underlying not only cell proliferation and cell death (Fig. 16), but also embryonic development, neuronal functions, or immunity [338]. Initially Jun kinase was identified as a member of the pp54 microtubule associated protein-2 kinases [339] and subsequently characterized as stress activated kinase preferentially phosphorylating and stimulating the activity of the transcription factor c-Jun [340]. The mammalian JNK proteins are encoded by three genes, which are alternatively spliced and give rise at least to 10 isoforms [341], JNK1 and JNK2 being more ubiquitous, whereas JNK3 is mainly found in the brain. JNK activation is regulated by phosphorylation on both threonine and tyrosine residues by a dual-specificity MAPK kinase (MAPKK). Two MAPKKs, MKK4 (also known as SAPK/Erk kinase (SEK1) or Jun kinase kinase (JNKK)), and MKK7, have been identified as JNK activators. The protein kinases that have been reported to act as MAPKKKs (MAP3Ks) for the JNK signaling pathway include the MEK/ERK kinase (MEKK) group, the MLK group, TPL-2, ASK1, and TAK1 [342] (see Fig. 18).

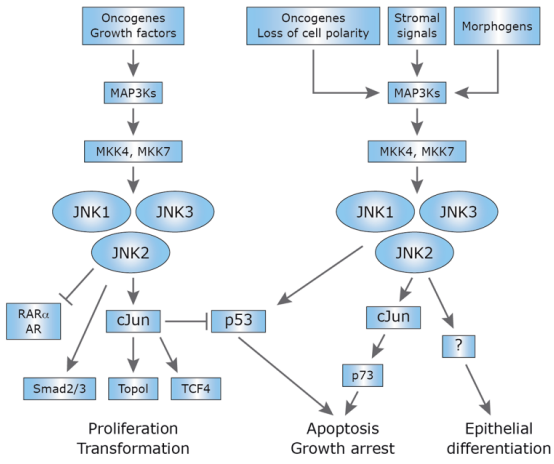

Figure 16. JNK-regulated pathways in cell transformation (redrawn from [343]).

A major JNK target is the transcription factor AP1, which is composed of Fos and Jun family members [340]. JNK was found to bind the NH2-terminal activation domain of c-Jun [344] and to phosphorylate c-Jun on Ser-63 and Ser-73 [345]. The phosphorylation of these specific

positions increases transcription activity of c-Jun [345, 346]. The oncogenic functions of JNKs are mostly based on the ability phosphorylate c-Jun, leading to activation of AP1 [340, 347].

3.7 Activator protein 1 (AP-1)

The AP-1 complex is composed of multiple homodimeric and heterodimeric protein complexes involving members of the Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1and Fra-2) families and it can heterodimerize also with other transcription factors such as members of the ATF/CREB and Maf/Nrl subfamilies [348-351]. These are structurally similar and functionally related basic leucine zipper (bZIP) proteins, forming the required dimers through the bZIP domain [352]. Dimerization brings the basic regions together and produces thus an adjoining interface able to interact with specific DNA sites [353]. The dimers recognize either TPA response (5'-TGA G/C TCA-3') or CRE elements (cAMP response elements) on DNA, with different affinity and sequence specificity [354]. AP-1 recognition sites have been found in many enhancer/promoter elements [355]. Once bound to DNA, the AP-1 complexes regulate transcription either by activating or repressing target gene transcription, each dimer differing in their activity depending on the cellular context. The AP-1-induced gene transcription is part of the regulation of a variety of critical cellular processes including cell proliferation, cell differentiation, cytokine production, apoptosis and oncogenesis [351, 356].

3.7.1 c-Jun

c-*jun*, the cellular homolog of a v-*jun* oncogene that was originally isolated from avian sarcoma virus 17 (ASV17) [357], encodes a 39 kDa nuclear phosphoprotein c-Jun [358, 359]. It is the most potent activator of the AP-1 family [340]. It has been suggested that c-Jun can function as a central link in the pathway coupling the signals from the cell membrane to the regulation of various genes and ultimately to the growth of cells. Various activating signals, like tumor promoters, growth factors or stress stimuli promote signal transduction pathways that can rapidly change the phosphorylation state of c-Jun and thus, significantly enhance the DNA binding and transcription activity of c-Jun [360-363].

There are five serine and threonine residues of c-Jun that can be phosphorylated [364]: three upstream of the DNA-binding domain and two within the transactivation domain (Fig. 17A). When the more C-terminal sites are dephosphorylated, the DNA-binding affinity of c-Jun is increased. The phosphorylation at Ser63 and Ser73 within the transactivation domain are important for the transcriptional activity of c-Jun [346, 365].

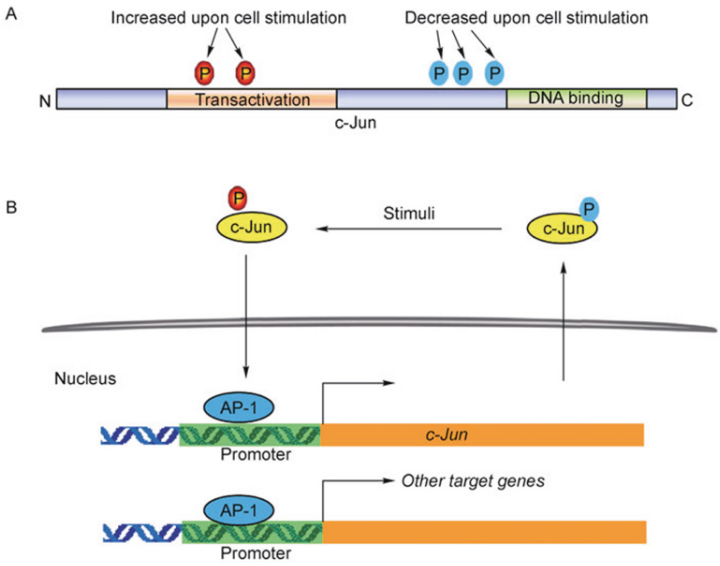

Figure 17. Phosphorylation regulates c-Jun expression and activity (A) and regulatory circuit between c-Jun and AP-1 (B) (from [366]).

In resting cells c-Jun is phosphorylated on the residues near to the DNA binding domain, and thus, silent. Upon stimulation these sites are dephosphorylated allowing interaction with TRE sequences in target genes. Serum and growth factor stimuli have been shown to induce AP-1 by activating the ERK subgroup of MAPKs through a twist via S6 kinase (Fig. 18). When glycogen synthase kinase 3 (GSK3) and casein kinase 2 (CK2) phosphorylate c-Jun at its C-termini, c-Jun is kept in an inactive, non-DNA-binding state [367]. While ERK can activate S6K, which in turn phosphorylates GSK-3 at serine-21, results this in its inactivation [368]. Hence, ERK, by acting through the S6K-GSK3 cascades, causes c-Jun C-terminal dephosphorylation near the DNA-binding domain and increases its DNA binding activity. The ERK1 and 2 have been shown to be able to bind and phosphorylate the C-terminal part of c-Jun directly, but this does not correlate with the c-Jun activation [369].

Activation of c-Jun through the JNK cascade is another important post-translational mechanism to control c-Jun [370] (Fig. 18). Activated JNKs translocate to the nucleus, where they bind to a specific region within the c-Jun transactivation domain and phosphorylate c-Jun on serines 63/73 and threonines 91/93 [371]. This N-terminal phosphorylation weakens also c-Jun's interaction with a histone deacetylase-containing inhibitory complex [372] and thereby its ability to activate transcription upon homodimerization or heterodimerization with c-Fos is potentiated [373-375].

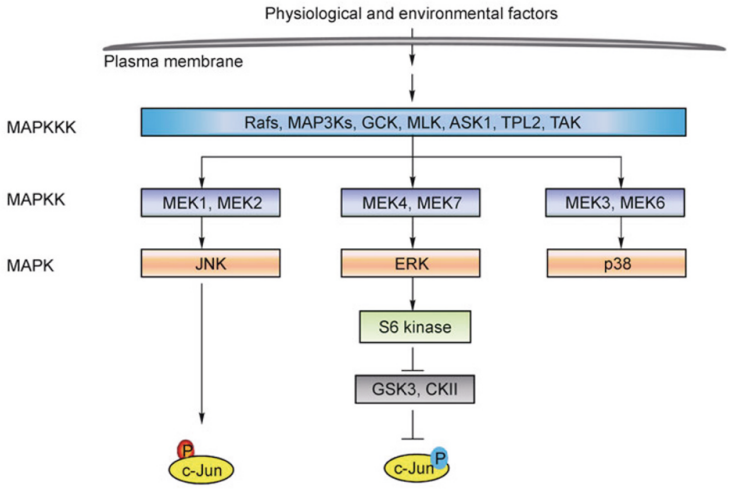

Figure 18. c-Jun can be phosphorylated by both JNK and ERK pathways (from [366]).

Interestingly, the specific binding of JNK to c-Jun explains why v-Jun, with conserved Ser63/73, but without the JNK binding domain, can resist the TPA-induced N-terminal phosphorylation [376, 377]. However, the lack of JNK docking domain also eliminates v-Jun binding to the repressor complex [372] that is suggested to contribute more to viral Jun oncogenicity than missing binding to JNK [378]. By this mean, viral Jun is disconnected from the tight controls regulating c-Jun; it is constitutively active and oncogenic.

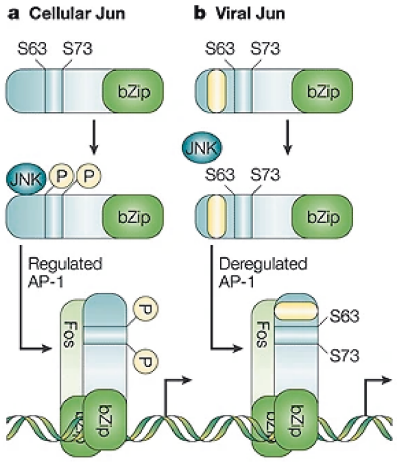

Figure 19. Deregulated Jun. a) Cellular Jun and b) viral version of Jun (from [377]).

The phosphorylated c-Jun may be a trigger for AP-1 activation, but as the phospho-c-Jun proteins are relatively unstable and exist only transiently in the cells, their role in maintaining the sustained AP-1 activity that is required for many biological activities has been questioned [379, 380]. Interesting observations have suggested the existence of a regulatory circuit, in which AP-1 activates the c-Jun gene expression that in turn further enhances AP-1, thereby potentiating its own gene activation [381-383] (Fig. 17B). By this kind of autocrine circuit c-

Jun can both prolong and amplify AP-1 activity. Accordingly, AP-1 activity is sustained and long-lasting biological outcomes can be maintained [381].

3.7.2 c-Jun in tumorigenesis

In view of the pivotal role of c-Jun in mediating the effects of various tumor promoting factors and the already known ability of v-Jun to transform, it was not surprising that uncontrolled expression of normal c-Jun protein as well transformed mammalian cells [384, 385]. Furthermore, it has been shown that c-*jun* as a single gene with normal coding potential can transform an immortalized rat fibroblasts [384] and over-expression of wild type c-Jun transforms chick embryo fibroblasts [386]. Moreover, c-Jun is required for transformation by other oncogenes such as *src*, *ras* and *raf* [348, 385, 387-390].

The activity of the transcription factor c-Jun is increased in several human cancers [340]. Interestingly, it has been also shown that c-Jun activating phosphorylation is a direct and central part in the development of colorectal cancer [391] and dysregulation of Jun by amplification and overexpression leads to highly aggressive human sarcomas [392].

4. Tumor angiogenesis

It is now well established and broadly documented that unrestricted growth of solid tumors beyond 2-3 mm in diameter is dependent upon vascularization, an event required for tissues to grant oxygen, nutrient and waste disposal [393, 394]. The early activation of angiogenic processes is compulsory for sustaining the tumor cell proliferation and vessel network functions. Later in the tumor development this serves as a route to disseminate cells to distant sites, promoting metastasis [395, 396].

4.1 Capillary structure

The basic structure of a vessel wall is depicted in Fig. 20 [397]. Newly emerging vessels consist of a tube of endothelial cells (Fig. 20a) that mature to capillaries, when basement membrane and pericytes within are covering the tubes (Fig. 20b) [398]. Because of their wall structure functioning as an endothelial barrier that is highly permeable to small water- and lipid-soluble molecules and their large surface area, capillary network forms the main site of exchange of nutrients, gases, and water between bloodstream and tissues [399]. The capillary endothelial layer can be naturally continuous, fenestrated or discontinuous, depending on organ or tissue type [398]. In comparison to capillaries, more mature arteries and veins have already an increased coverage layer of mural cells (Fig. 20c). However, extravasation of cells and macromolecules from the blood typically occurs from these postcapillary venules [400]. The walls of larger vessels differ from the walls of arterioles and venules by consisting of three clearly specialized layers (Fig. 20d). Due to the smooth muscle cells within and elastic laminae, this type of vessels have already more defined diameter and better control over blood flow [397, 398].

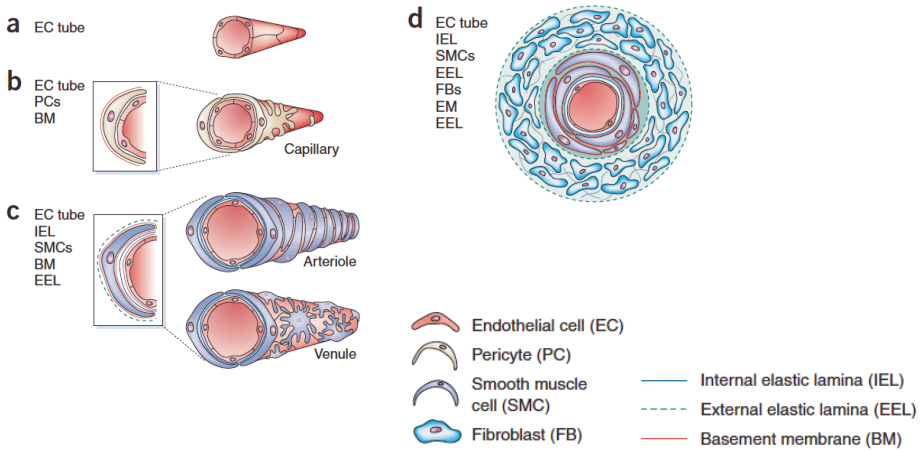

Figure 20. Wall structures of growing versus mature vessels. a) Nascent vessel, b) Capillaries consist of endothelial cell tube surrounded with basement membrane with a thin layer of pericytes embedded in it. c) Mural cells, like pericytes add a stabilizing layer to capillaries. d) The larger vessels wall are composed of three specialized layers that provide better control of blood flow (adapted from [397]).

Normal physiological angiogenic responses follow a structural hierarchy: perivascular cells lose their association with the endothelium, endothelial junctions become leaky and finally the basement membrane is modified [401, 402]. Tumor angiogenesis, instead, is dysregulated as it does not follow any organized process and produces poorly formed blood vessels with multiple abnormalities. For example, a hierarchical assembly is missing, vessels have irregular diameters, they are highly tortuous, randomly branched and endothelial barrier function is defective [397, 402]. Tumor vessels can have abnormal organization of pericyte coat and basement membrane might be incomplete [403, 404]. Although the molecular control systems leading to these abnormalities are not fully understood, it is assumable that they are a result of the bizarre tumor microenvironment created by deregulated growth factors combined with hypoxic and metabolic disturbances.

4.2 Mechanisms underlying angiogenesis

To date, several mechanisms have been shown to be employed in the neovascularization process of the tumors, some originally identified to contribute normal and physiological angiogenesis [405]. It seems, however, in pathological neovascularization the mechanisms are not mutually exclusive and act somewhat simultaneously. These mechanisms are depicted and reviewed in the work of Ronca et al [394] (Fig. 21).

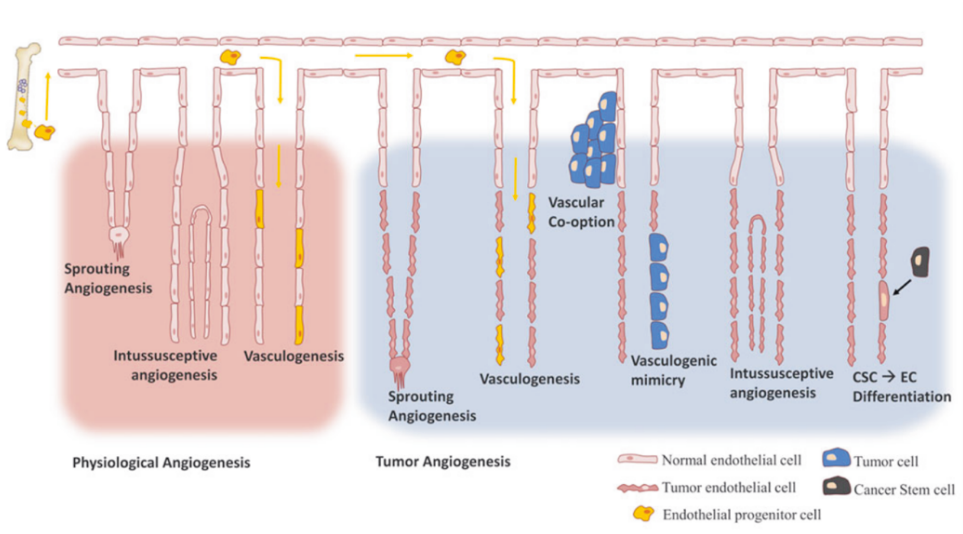

Figure 21. Physiological and tumor neovascularization (adapted from [394]).

4.2.1 Vasculogenesis

Vasculogenesis together with sprouting angiogenesis are prototypical mechanisms of vascularization, known to form and remodel vessels during development, and to get reactivated during tumor progression. Vasculogenesis describes a process, where vascular progenitor cells differentiate and proliferate within a previously avascular tissue, and then merge into a crude tubular network [406]. During angiogenic remodeling the initial network is modified, interconnecting the branching patterns, maturing vessels, and finally, forming functional vasculature [407].

4.2.2 Sprouting angiogenesis

A process of angiogenic sprouting refers to a multistep model in which sprouting from existing vessels creates new capillary vessels into a previously avascular tissue [394, 408]. It is an intensive interplay between extracellular matrix components, cells, and soluble factors [394, 409]. At first, proangiogenic factors (like VEGF) activate endothelial cells toward invasive and motile behavior that induces destabilization of the endothelial-pericyte contacts, leading to the remodeling of the basement membrane. This in turn, allows the endothelial cells at the cell's front or leading-edge to form filopodia [410] and migrate through the extracellular matrix. The cells at the leading-edge (or migrating tip cells) are followed by highly proliferative endothelial cells located at the stalk of the angiogenic sprout. Vessels formed by sprouting are again initially immature and must further develop. They are the stalk cells that are responsible for the tube and branch formation and for the recruitment of pericytes for stabilization.

4.2.3 Intussusceptive angiogenesis (vessel splitting)

The concept of vessel intussusception or splitting angiogenesis was originally reported by Caduff et al [411]. In this type of vessel formation, the wall of pre-existing capillary extends

into the lumen and splits a single vessel in two. The proposed mechanisms involved in the formation of split vessels is described in detail in ref [412].

Interestingly, during intussusception, neither endothelial cell proliferation or large basement membrane degradation is required. This is metabolically less demanding and occurs more rapidly compared to sprouting angiogenesis, and can, thus, respond faster to an oxygen demand in a growing tissue [413]. Many human tumors display vessel splitting (see refs in [394]) and angiogenic switch from sprouting to intussusceptive angiogenesis has been stated in relapsing tumors after irradiation or anti-angiogenic therapy, and may represent an adaptive tumor-protective response to treatment [394, 414].

4.2.4 Vascular co-option

In vascular co-option or vessel “hijacking” tumor cells surround host vessels and encompass them to their own purposes [415]. This eliminates the need for neoangiogenesis (tumors are therefore called nonangiogenic), so the co-opted vessels lack the typical abnormal features of tumor vasculature, making them less prone to respond to antiangiogenic therapy [394, 416]. Mainly the tumors in the highly vascularized tissues (like liver, lung and brain tissues) have been shown to exploit vascular co-option as a blood supply [394].

4.2.5 Endothelial progenitor cells

Endothelial progenitor cells are residing in a stem cell niche in the bone marrow. From there they are mobilized to the peripheral blood and the tumor sites by various cytokines and proteolytic enzymes [394]. On the site of tumor vascularization endothelial progenitor cells differentiate into endothelial cells resulting in angiogenesis [417, 418]. Increasing amounts of the endothelial progenitor cells have been observed also in many cancers [419, 420]. Even though the concept [417] that endothelial progenitor cells are not only involved in embryonic development but also contribute to adult vasculogenesis has been intensively studied, its role in tumorigenesis has remained unclear [394]. This could be due to the appropriate definition for endothelial progenitor cells, their origin and other characteristics that is still missing.

4.2.6 Cancer stem cells

Cancer stem cells are defined as rare immortal cells within a tumor that can both self-renew by dividing and give rise to many cell types that comprise the tumor [421]. Recent studies have suggested that cancer stem cells could also differentiate to endothelial cells and provide endothelial lining in human cancers [422, 423]. This hypothesis was supported by the finding that the same mutation of the original tumor cell could be found in the subpopulation of endothelial cells [422].

4.2.7 Vasculogenic mimicry

The term vasculogenic mimicry has been described originally as very pluripotent cancer cells’ ability to form extracellular matrix-rich networks in 3D culture [424]. They are called vasculogenic even though these pathways are not formed of the pre-existing vessels, but are able to distribute plasma and may contain red blood cells. However, the channels are not

blood vessels, they just mimic the function of vessels [425]. The channels were found to be lined with tumor cells instead of endothelial cells and were rich in laminin [424, 426].

To date, vasculogenic mimicry is distinguished in the tumor samples using immunohistochemistry staining positive for both CD31 and periodic acid Schiff (PAS) stain, CD31 being a marker for endothelial cells and PAS to identify extracellular matrix. Two types of vasculogenic mimicry has been described in aggressive tumors: the tubular type is composed of non-endothelial cell-lined blood tubes, confusing them with morphologically similar endothelial cell-lined blood vessels [427] and the patterned matrix type, comprising of a basement membrane rich in fibronectin, collagens and laminin, and where the membrane is surrounded directly by tumor cells [428].

The initial description of "highly patterned vascular tubes formed by aggressive uveal melanoma cells without the endothelial cell lining" by Maniotis et al [424], was received by skepticism [429, 430]. Since then, however, vasculogenic mimicry has been described in numerous types of cancers, including breast, lung, colon and ovarian carcinoma [431]. Further, its presence in melanomas [432] and other cancers [433] has been shown to associate with a poor prognosis. Moreover, it is suggested that both angiogenesis and vasculogenic mimicry are coordinately used in tumor tissues [434]. In tumor areas, where vasculogenic mimicry is part of the functional plasma circulation, cancer cells from the tumor cell-lined channels have a facilitated access via endothelial-lined vessels out of the tumor into the blood circulation, contributing thus to invasion and metastasis (Fig. 22).

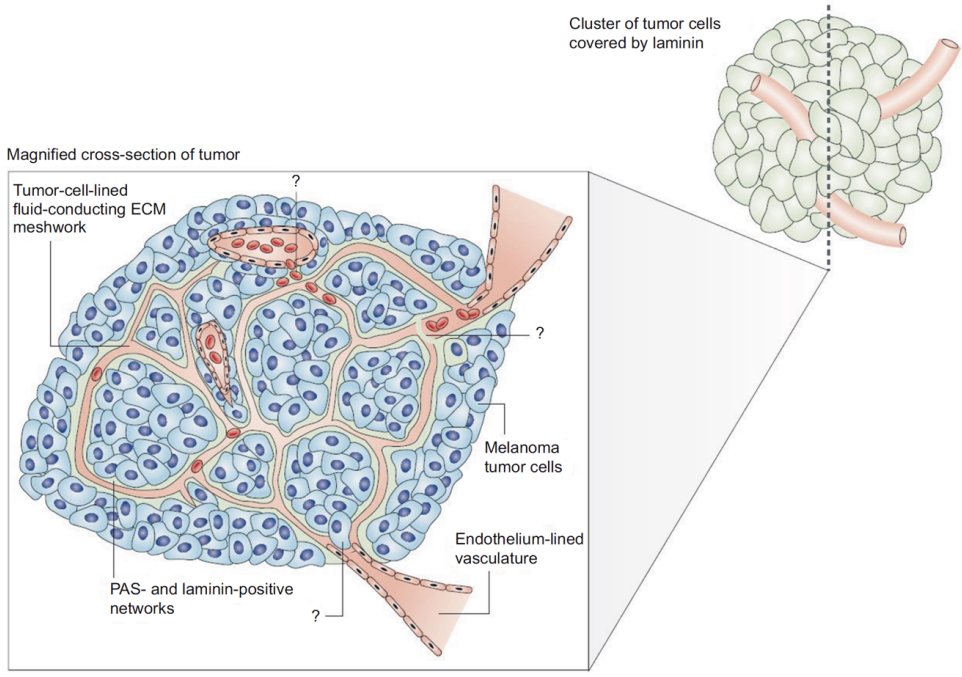

Figure 22. Melanoma and its extracellular matrix containing both endothelial and tumor-cell-lined fluid-containing channels (from [435]). Melanoma (blue cells) contain endothelium-lined vessels (pink) in close contact to fluid-containing matrix formed by tumor cells (green). Tumor cell-induced remodeling of the vasculature can lead to the accumulation of red blood cells (red) outside of the vasculature. The biological implications or the relationship of the systems (marked "?") still need to be clarified.

Epithelial-mesenchymal transition (EMT) or cancer stem cells (CSC) have been suggested as possible mechanisms for the development of vasculogenic mimicry patterns [436] and the list of contributing protein factors (such as VE-cadherin, matrix metalloproteinases, ephrin type-A receptor 2, and NOTCH proteins) is growing [431, 435]. Despite remarkable advances, the key mechanisms of vasculogenic mimicry have remained ambiguous.

4.2.8 Mosaic vessels

Tumor vessels that constitute of both endothelial and cancer cells that are in direct contact with the vessel lumen, have been described as mosaic [437, 438]. Mosaic vessels have been identified in experimentally induced mouse xenocrafts and human biopsies of colon carcinoma [439] and more recently in MYCN amplified neuroblastomas [440] and glioblastomas [422, 423]. The association of mosaic vessels to very aggressively growing tumors could be an indication for invasive and metastatic tumor growth [439, 441]. Also, the participation of tumor cell on the vessel lining would have important consequences for therapeutics design that targets the tumor vasculature.

Di Tomaso et al [441] suggest several possibilities how mosaic vessels could originate. “First, endothelial migration during rapid vessel growth occurs without sufficient endothelial proliferation to complete the endothelial lining. Second, endothelial cells shed from the vessel lining, leaving underlying tumor cells exposed. Third, surrounding tumor cells invade the vessel wall and displace endothelial cells from the lining. And fourth, mosaic regions are lined by abnormal endothelial cells that do not express common endothelial markers, and cannot be detected”. Improved detection of endothelial cells, combined with consensus on the endothelial markers and with a better understanding of the plastic nature of tumor cells together with their interaction with surrounding matrix could bring clarification to this complex system in the future.

Additionally, an intriguing possibility has been proposed that the PAS- and laminin-rich channel network described above, after being an early survival mechanism for the acute needs of the growing tumor, could be replaced by endothelial cells from the nearby angiogenic vessels, the bone marrow or from the circulating endothelial precursor cells that would later cover the exposed tumor cells on the vessel wall [417, 426, 438, 442]. Mosaic vessels would then in this case be a temporary phenomenon, on the way towards more stable tubular structures.

4.3 Angiogenic switch

The induction of tumor angiogenesis, termed the “angiogenic switch” is a hallmark of cancer [281]. It is driven by the growing tumors’ requirement for oxygen supply and waste removal [393, 394]. The observations that tumors implanted into an avascular region were able to elicit the ingrowth of new capillaries, suggested that they release diffusible factors, able to activate a quiescent vasculature [443]. This has led to a finding of several tumor angiogenesis stimulating factors, such as vascular endothelial (VEGF), fibroblast (FGF), platelet-derived (PDGF) and epidermal growth factors (EGF) [444]. Factors inhibitory to angiogenesis are an equally important component of the switch. The significance of these negative regulators of angiogenesis became obvious in experiments that compared cell lines containing or missing

the tumor suppressor gene [445, 446]. The nontumorigenic cell line released high levels of a potent angiogenesis inhibitor, while tumorigenic cell line released much less [446]. In general, the levels of activators and inhibitors comprising the angiogenic balance, determine whether an endothelial cell will be in quiescent or an angiogenic state is induced. It is assumed that the changes in this balance are the triggers of the angiogenic switch.

In physiological and normal angiogenesis, the balance between pro- and anti-angiogenic signals is tightly regulated: new vessels mature rapidly, become stable and return back to quiescence. Normal vasculature is well organized into definitive venules, arterioles and capillaries, in which the metabolic needs of the tissue determine the vessel density (Fig. 23 left). However, after angiogenic switch, tumor blood vessels remain active, continue uncontrolled growth and result in irregularly shaped, dilated and tortuous vascular networks with numerous dead ends [397, 444] (Fig. 23 right). Overexpression of growth factors, like vascular endothelial growth factor (VEGF) can make the vessels leaky and tumors hemorrhagic [447]. Also, perivascular cells that have been embedded in endothelium, become less abundant or more loosely attached [403].

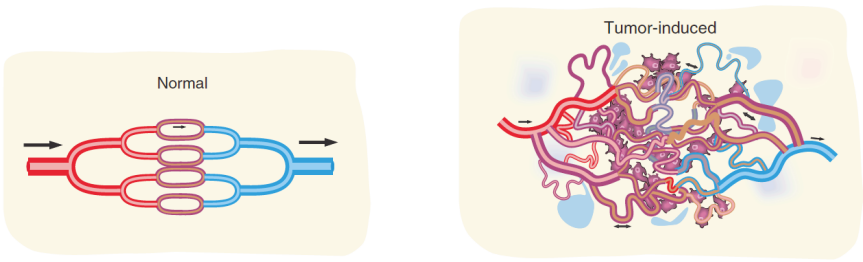

*Figure 23. Highly simplified view of normal versus tumor-induced vascular bed (*adapted from [397]). a) Normal vascular bed has a regular appearance and physiological angiogenic responses follow a structural hierarchy. b) Activated angiogenesis and vessel remodeling result in abnormal vascular bed leading to chaotic blood flow in tumors.

4.3.1 Proangiogenic molecules

Vascular endothelial growth factor (VEGF) (also termed VEGF-A) belongs to a large family of structurally related mitogens [448] and is a major regulator of normal and abnormal angiogenesis associated with tumor growth [449]. The physiological effects of VEGF are mediated through binding to two homologous transmembrane tyrosine kinase receptors, VEGF receptor-1 and -2 that are expressed on vascular endothelial cells and undergo dimerization upon ligand binding [450]. Dimerization triggers signal transduction by promoting the phosphorylation of the receptors, and thus, is activating the downstream signaling.

VEGF induces angiogenesis directly by mitogenic effect on endothelial cells [451]. *In vitro* assays showed that VEGF induced endothelial cell invasion into the matrix, where they formed capillary-like tubules. VEGF elicits also non-mitogenic responses on vascular endothelial cells. By inducing anti-apoptotic signals, VEGF is required not only for proliferation but also for the survival of endothelial cells, thus being important for the viability of immature vasculature [452]. Additionally, VEGF can increase vascular permeability, leading to leakage of plasma

proteins and development of an extravascular matrix, further enhancing the remodeling of the environment, favorable for endothelial cell growth [450].

Experimental evidence supports the association of increased VEGF expression to the activation of several oncogenes like K-ras, H-ras, v-src, HER2, EGFR, FOS or to the loss of tumor suppressor p53 [453]. Also, an increased expression of VEGF has been demonstrated in several human cancer types that correlates with invasiveness, vascular density, metastasis, recurrence, and prognosis [454].

4.3.2 Antiangiogenic molecules

Thrombospondin-1, the first discovered member of a family of multifunctional extracellular matrix (ECM) proteins (TSPs 1-5), is the inhibitory activity described above that was purified and shown to be a truncated form of thrombospondin-1 (TSP-1) [455]. TSP-1 is a 450 kDa homotrimeric glycoprotein that can be divided into structural domains, each domain having multiple receptors or molecules to interact in the cell (Fig. 24) [456].

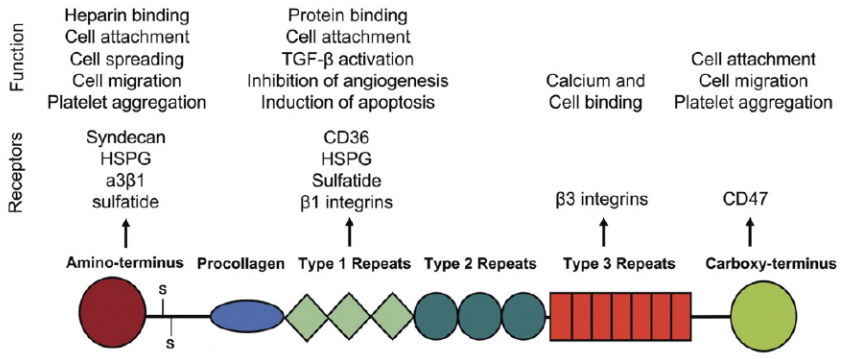

Figure 24. Structural and functional domains of TSP-1 (from [456]).

Each subunit of TSP-1 consists of amino (N)- and carboxy (C)-terminal globular domains, a region of homology with procollagen and three types of repeated sequence motifs designated types 1-3 repeats (Fig. 24). The varied functions of TSP1 are determined by the interactions between its structural domains and multiple cell surface molecules [456]. The sequence similarities between TSP-1 and TSP-2 suggests that they bind to same molecules on the cell surface and thus, functions mediated by these receptors may be common to both proteins [457].

TSP-1 has been shown to regulate very diverse and even opposite cellular processes: TSP-1 can be a promoter of the migration for vascular smooth muscle cells, but it is, on the other hand, an effective inhibitor of endothelial cell migration [458, 459]. By binding to matrix proteins such as fibronectin and collagen and by modulating the activity of extracellular proteinases such as matrix metalloproteinases (MMPs) and plasmin, TSP-1 can be considered as one of the key players in modulation of the tumor microenvironment [460].

It is still controversial, what is the exact biological function of TSP-1 in tumorigenesis and angiogenesis, as some studies have found it to contribute to cell transformation [458, 461]

and promote neovascularization [462]. TSP-1 downregulation, instead, has been found in several primary tumors [458] and in cells transformed *in vitro* by Ras [463], v-Src [464], v-Myc [465], ODC [466], and c-Jun [467]. In a present view TSP-1 is recognized as an endogenous inhibitor of angiogenesis and as TSP-2 has equivalent domain structure, it is also shown to be a potent inhibitor of angiogenesis [457].

5. Matrix modulation for angiogenesis and invasion

During angiogenesis extracellular matrix (ECM) needs to be degraded and modulated for endothelial cell migration taking place [396, 468]. Also, conversion to an invasive phenotype involves similar events of remodeling, including the attachment of the tumor cells to the basement membrane via cell surfaces receptors, and the secretion of enzymes that degrade the basement membrane and the underlying stroma [468-470]. Matrix metalloproteinases (MMPs) participate by their protein degrading ability in the matrix disruption, tumor neovascularization, and metastasis, whereas tissue inhibitors of metalloproteinases (TIMPs) downregulate the activity of MMPs, reviewed in [471, 472].

5.1 Matrix metalloproteinases (MMPs)

MMPs belong to a large family of endopeptidases that can cleave any component of ECM [471]. Several different MMPs has been characterized in mammals that are divided in soluble (e.g. MMP-2 and MMP-9) and membrane-type MMPs (MT-MMPs) and classified further in subfamilies based on their structure [471]. Mammalian MMPs share a conserved domain structure that consists of a catalytic domain and an autoinhibitory pro-domain (Fig 25). The pro-domain contains a conserved cysteine residue that in turn coordinates the zinc ion at the active site to inhibit catalysis. MMP-2 and MMP-9 have also fibronectin type II repeats that bind to collagens inserted into the catalytic domain (Fig. 25). MMPs are synthetized as latent proenzymes and are activated mainly outside the cell by other activated MMPs or serine proteases [473]. MMP-2 instead, is activated at the cell surface through a unique multistep pathway involving MT1-MMP and TIMP-2 [474]. Removal of an amino-terminal pro-domain is required for the production of active (62 and 59 kDa) enzyme forms from the 72 kDa-sized inactive proenzyme form [475].

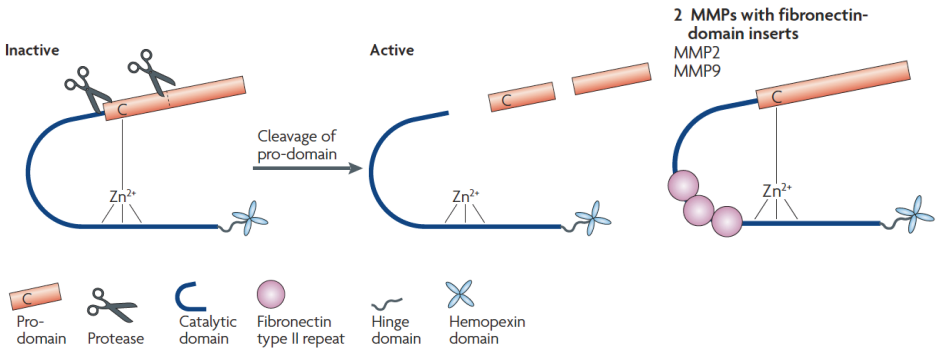

Figure 25. Schematic structure of MMPs. MMP2 and MMP9 share a conserved domain structure (adapted from [476]).

The role of MMPs in tumor angiogenesis and cancer progression is strongly supported by the fact that the expression and activity of MMPs have been found increased in almost every type of cancer, correlating with advanced tumor stage, increased invasion and metastasis, and shortened survival [471]. Specifically, MMP-2 and MMP-9 have been implicated in angiogenesis regulation [472, 477, 478].

5.2 Tissue inhibitors of metalloproteinases (TIMPs)

The synthesis, secretion and activation of the MMPs together with the synthesis and secretion of their inhibitors, TIMPs, are key regulatory steps in the remodeling of ECM in both normal and pathological processes, like angiogenesis and tumor invasion [479]. Four mammalian TIMPs (TIMP-1-4) have been identified. They are two-domain molecules, of which the N-terminal domain performs the MMP-inhibitory function [480] by binding to MMPs. TIMPs have various biological activities including the modulation of cell proliferation, cell migration and invasion, anti-angiogenesis, and anti- and pro-apoptosis [479]. These activities may partially arise from metalloproteinase inhibition, but many of them have been shown to be independent of MMP inhibition [479, 481]. The emerging explanation for these contrasting roles is that TIMPs function seems to depend on the context so that the tissue microenvironment determines finally the mechanism of action.

5.3 Tenascins

Tenascin-C (TN-C) is a hexameric, multimodular protein (Fig. 26) that belongs to a highly conserved family of large glycoproteins found in the extracellular matrix [482, 483].

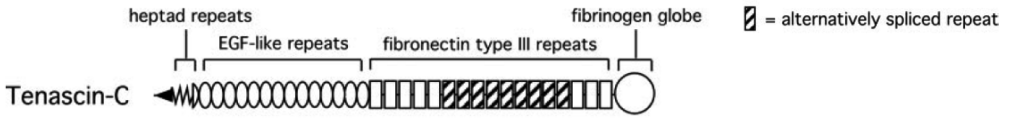

Figure 26. Schematic representation of tenascin-C (adapted from [483]). An amino-terminal oligomerization region is followed by consecutively arranged domains of heptad repeats, EGF-like and fibronectin type III repeats, and a fibrinogen globe. Alternative splicing and protein modifications are rich sources for numerous TN-C forms.

The multimodular structure (Fig. 26) enables the tenascins to interact with a high number of highly diverse ligands leading to complex cell interactions. TN-C is the best known for its function as an adhesion-modulating extracellular matrix protein through binding to fibronectin, but TN-C has been shown to have also important and even opposite effects, depending on the context, on cell proliferation, migration, angiogenesis, survival, transformation, metastasis and immunosurveillance [484]. The normal TN-C expression in healthy organism is sparse [485, 486], but it can be rapidly induced in response to pathological stress, like upon infection and inflammation [487], and in human cancers [484, 488].

AIMS OF THE STUDY

The aim of this work was to engineer rodent fibroblasts to overexpress polyamine biosynthetic enzymes ornithine and S-adenosylmethionine decarboxylases in sense and antisense orientations at constant high levels and to study the effects of their activity on cell growth and morphology, and their potential tumorigenicity in immunocompromised mice.

Further, once these enzymes were found to be transforming, we wanted to compare signal transduction pathways in ODC-, AdoMetDC, c-Ha-*ras*^{Val12} - and v-*src*-oncogene transformed cells, and search for potential points of convergence that could be common in various oncogenic transformation models.

MATERIALS AND METHODS

The methods used in the study are summarized in Table 2 and detailed description of the methods can be found in the corresponding original publications. Cell lines and vectors, and exact references to them are described in Table 3. Table 4 lists the antibodies used in all four publications.

Table 2. Methods

Method	**Used in**
Cloning	I, III, IV
Expression vectors	Table 3
Cells and transfections	
Cell lines	Table 3
Stable transfections	I, III, IV
Transient transfections	III, IV
Culturing cells	I, II, III, IV
Cell growth and tumorigenicity assays	
Cell proliferation in high and low serum	I, IV
Soft-agar growth	I, III, IV
Tumorigenicity / *nude* mice	III, IV
Analysis of biomolecules	
DNA analyses	
Southern blot	I
RNA analyses	
Northern blot	I, II, III
Slot blot	I
Microarrays	
cDNA microarray	IV
Oligonucleotide microarray	IV
RT-PCR	IV
Polyamine analysis	I, III
Analysis of proteins	
Western blotting	I, II, III, IV
Antibodies	Table 4
Cell lysates	I, II, III, IV
Nuclear fractions	II, III
Protein assay	I, II, III, IV
Immunoprecipitation	II, III
Receptor binding assay	II
Enzymatic assays	
ODC activity	I, III
AdoMetDC activity	III
MAPK activity	II, III
JNK activity	II, III
Src kinase activity	II
Histochemistry	
Hematoxylin & eosin staining	IV
Actin filament staining	III
CD31 staining	IV
Laminin staining	IV
Tenascin staining	IV
Functional assays	
Matrigel invasion	IV
Endothelial cell migration	IV
Conditioned media	IV
Gelatin zymography	IV

Table 3. Cell lines and vectors

Name	Parental cell line/ named to	Vector	Reference for vector	Insert	Reference for cell line / insert	Used in
NIH3T3	Mouse fibroblasts	-		-	ATCC CRL-1658	I, II, III, IV
N1	NIH3T3	pSV2neo	ATCC 37149	-	[135]	I, II, IV
E2	NIH3T3	pEJ6.6	[489]	c-Ha-*ras*Val12	[135]	II
E4	NIH3T3	pEJ6.6	[489]	c-Ha-*ras*Val12	[135]	I, II, IV
pLTR-S	NIH3T3/Odc	pLTRpoly	ATCC 77109	1.8kb ODC-insert from pODC10/2H sense	[490]	I, II
pLTR-AS	NIH3T3	pLTRpoly	ATCC 77109	1.8kb ODC-insert from pODC10/2H antisense	[490]	I
pMAM-S	NIH3T3	pMAMneo	Clontech	1.8kb ODC-insert from pODC10/2H sense	[490]	I
pMAM-AS	NIH3T3	pMAMneo	Clontech	1.8kb ODC-insert from pODC10/2H antisense	[490]	I
pAPr-S	NIH3T3	pHßApr1neo	[491]	1.8kb ODC-insert from pODC10/2H sense	[490]	I
pAPr-AS	NIH3T3	pHßApr1neo	[491]	1.8kb ODC-insert from pODC10/2H antisense	[490]	I
ODC-n	Derived from pLTR-S cell-induced tumors in nude mice	pLTRpoly	ATCC 77109	1.8kb ODC-insert from pODC10/2H sense	[466]	II, IV
RSVLA29 Rat-1	Rat-1	-	-	tsLA29src	[492]	I, II
R2		-	-	ts339src of RSV-B77	[493]	I
Rat-1	Rat fibroblasts	-	-	-	ATCC CRL-2210	II, III
Rat-1 neo control	Rat-1	pLTRpoly, pSV2neo	ATCC 77109, ATCC 37149	-	-	III
Rat-1 Amdc-s	Rat-1	pLTRpoly, pSV2neo	ATCC 77109, ATCC 37149	1.65kb AdoMetDC insert from pSAMh1 sense	[120]	III
Rat-1 Amdc-as	Rat-1	pLTRpoly, pSV2neo	ATCC 77109, ATCC 37149	1.65kb AdoMetDC insert from pSAMh1 antisense	[120]	III
4N	NIH3T3	pLTRpoly, pSV2neo	ATCC 77109, ATCC 37149	-	-	III, IV
AMDC-S	NIH3T3	pLTRpoly, pSV2neo	ATCC 77109, ATCC 37149	1.65kb AdoMetDC insert from pSAMh1 sense	[120]	III, IV
AMDC-AS	NIH3T3	pLTRpoly, pSV2neo	ATCC 77109, ATCC 37149	1.65kb AdoMetDC insert from pSAMh1 antisense	[120]	III
pcDNA3	4N	pcDNA3, pBabe-puro	Invitrogen, [494]	-		III
DN SEK1	4N	pcDNA3, pBabe-puro	Invitrogen, [494]	SEK1(AL)	[495]	III
DN JNK1	4N	pcDNA3, pBabe-puro	Invitrogen, [494]	FLAG-JNK1(APF)	[496]	III
pcDNA3	AMDC-S	pcDNA3, pBabe-puro	Invitrogen, [494]	-	-	III
DN SEK1	AMDC-S	pcDNA3, pBabe-puro	Invitrogen, [494]	SEK1(AL)	[495]	III

DN JNK1	AMDC-S	pcDNA3, pBabe-puro	Invitrogen, [494]	FLAG-JNK1(APF)	[496]	III
pCMV	4N	pCMV, pZeoSV2	Invitrogen	-	-	III
TAM67	4N	pCMV, pZeoSV2	Invitrogen	TAM67	[497]	III
pCMV	AMDC-S	pCMV, pZeoSV2	Invitrogen	-	-	III
TAM67	AMDC-S	pCMV, pZeoSV2	Invitrogen	TAM67	[497]	III
4N + vector	4N	pBabe-puro	[494]	-	-	IV
4N + TSP-1	4N	pBabe-puro	[494]	mouse TSP-1 insert from pJDM	[498]	IV
Amdc-s + vector	AMDC-S	pBabe-puro	[494]	-	-	IV
Amdc-s + TSP-1	AMDC-S	pBabe-puro	[494]	mouse TSP-1 insert from pJDM	[498]	IV
HT-1080	Human fibrosarcoma cell line	-	-	-	-	IV
MDA-MB231	Human breast cancer cell line	-	-	-	-	IV

Table 4. Antibodies

Target		**Clone or *Cat. #***	**Source**	**Method ***	**Publication**
P-Tyrosine	pAb	-	[499]	WB	I
PDGFR-α	pAb	R7	C-H Heldin & L Claesson-Welsh	WB	II
PDGFR-β	pAb	R3	C-H Heldin & L Claesson-Welsh	WB	II
p91	pAb	c	JE Darnell Jr.	WB, IP	II
P-Tyrosine	mAb	4G10	Upstate Biotechnology Inc.	WB, IP	II
Nck	mAb	*05-160*	Upstate Biotechnology Inc.	WB	II
PLCγ-1	mAb	*05-163*	Upstate Biotechnology Inc.	WB	II
GAP	pAb	*06-157*	Upstate Biotechnology Inc.	WB	II
SHC	pAb	*06-203*	Upstate Biotechnology Inc.	WB	II
Sos-1	pAb	*07-337*	Upstate Biotechnology Inc.	WB	II
SHPTP2/SYP	pAb	*06-118*	Upstate Biotechnology Inc.	WB	II
MAP kinase R2	pAb	Erk1-CT	Upstate Biotechnology Inc.	WB	II
JAK1	whole antisera	*06-272*	Upstate Biotechnology Inc.	WB	II
JAK2	whole antisera	*06-255*	Upstate Biotechnology Inc.	WB	II
PI3K	whole antisera	p85	Upstate Biotechnology Inc.	WB	II
P-Tyrosine	mAb	PT-66	Sigma	WB	II
Erk1/Erk2	mAb	Z033	Zymed Laboratories	WB	II, III
v-Src	mAb	327	Oncogene Science	IP	II
Raf-1	pAb	C-12	Santa Cruz Biotechnology	WB	II
c-Jun/AP-1	pAb	N	Santa Cruz Biotechnology	WB	II
JNK1	pAb	C17	Santa Cruz Biotechnology	IP	II
JNK1	pAb	FL	Santa Cruz Biotechnology	WB	III
P-c-Jun	pAb	Ser73	New England Biolabs Inc	WB	III
MKK4/JNKK1/SEK1	pAb	*M-0422*	Sigma-Aldrich	WB	III
c-Jun/AP-1	pAb	Ab-1	Calbiochem-Novabiochem	WB	III
CD31	mAb	MEC 13.3	BD Pharmingen	IHC	IV
Laminin	pAb	*L9393*	Sigma-Aldrich	IHC	IV
TN-C	mAb	Mtn15	[500]	WB	IV
VEGF	pAb	147	Santa Cruz Biotechnology	WB	IV
MMP-2	pAb	*AB808*	Chemicon International	WB	IV
TSP-1 (Ab4)	mAb	Ab6.1	NeoMarkers	WB	IV
MTI-MMP	mAb	113-5B7	Calbiochem-Novabiochem	WB	IV

* WB=Western blotting, IP=immunoprecipitation, IHC=immunohistochemistry

RESULTS & DISCUSSION

1. ODC activity is critical for cell transformation

1.1 *ODC* gene cloned into three different expression vectors in sense and antisense orientations

To be able to study the role of ODC in cell transformation, the human ODC cDNA (an 1.8 kb *Eco*RI-insert of the plasmid pODC10/2H [490]) was cloned in sense and antisense orientations into three different expression vector constructs (pHβAPr1neo [491], pLTRpoly [501] and pMAMneo (Clontech)). The construct in sense orientation was expected to express ODC over the natural, transient and tightly regulated levels at constant and high level, enabling observations on its effect on cell morphology. The construct in antisense orientation was foreseen to pair with its own endogenous ODC RNA and thus, to prevent it from being translated into a functional protein.

1.2 Stable transfections in NIH3T3 and R2 cells

NIH3T3 mouse fibroblasts were transfected with pHβApr1neo-S (sense orientation designated pAPr(S) cells) or pHβ–Apr-1-neo-AS (antisense orientation pAPr(AS) cells) constructs and compared to those transfected with pLTRpoly-S or -AS constructs (pLTR(S) and pLTR(AS) cells, respectively) and pMAMneo-S and -AS constructs (pMAM(S) and pMAM(AS) cells, respectively). pAPr(S) and pLTR(S) cells were growing more rapidly than the non-transfected cells and the growth rate of pAPr(AS) and pLTRpoly(AS) cells was reduced in comparison to their normal counterparts. To avoid problems caused by clonal variation, uncloned cell pools were used to produce the data presented.

Rous sarcoma virus-infected rat fibroblast R2 cells carrying a temperature inducible *src*-gene mutant were also transfected with both antisense constructs, pHβ–Apr-1-neo-AS and pLTRpoly-AS.

1.3 Confirmation of genomic integration and expression of human ODC by DNA and RNA analyses, and ODC-activity assays

The integration of ODC gene into the genome of NIH3T3 cells was confirmed by Southern blotting. The analyses showed that the pAPr(S) and pAPr(AS) cells had ODC-construct integrated in a lower copy number and with different integration sites than in the pLTRpoly/ODC constructs containing cells (data not shown). However, in Northern and slot blot assays the pAPr-ODC mRNA species were readily seen (I: Fig 3c and d). The size of the chimeric ODC mRNA extracted from the pHβApr1neo/ODC-transfected cells was 2.3 kb, as predicted (1.8 kb ODC insert size + 500 bp from the vector) and indistinguishable from the size of the endogenous mouse mRNA. Other mRNAs were detected in the cells expressing the antisense construct under the β-actin promoter (I: Fig 3c). The larger mRNA is speculated to result from unsplicing of the intervening sequences and the smaller is probably a result of alternative splicing or rearranged RNA. Notably, the antisense sequence of ODC cDNA contains a polyadenylation signal after 145 nucleotides, which results in such short RNAs (≤0.2 kb) difficult to capture by EtOH precipitation and poorly retained in the filter membrane. This resulted in very faint signals in Northern blotting. The slot-blot assay in which the membrane

immobilized RNA is hybridized with a single-stranded oligonucleotide probe, specific to this short sequence, circumvented the problem. Both the ODC antisense constructs in pLTRpoly and pHβ-Apr-1-neo gave rise to this short RNA sequence (I: Fig 3d). The three different expression plasmids containing the ODC gene proved to have promoters of different strength.

ODC activity measurements showed the activity in pAPr(S) cells to be 40-50% of that in pLTR(S) cells, where the ODC activity was elevated 50- to 100-fold over the control NIH3T3 cells, grown both in the absence or presence of serum (I: Fig. 3e and data not shown). The ODC activity in pAPr(AS) and pLTR(AS) cells, in turn, was decreased to almost undetectable levels (I: Fig. 3e).

1.4 Morphologic transformation

Transfection of NIH3T3 cells with a pLTRpoly/ODC in sense orientation elicited a complete morphological transformation. The cells had acquired an ability to grow over each other's without contact inhibition that is typical for transformed cells. Both in monolayer cultures and in soft agar, pLTR(S) cells formed multiple foci (I: Fig. 1b,e). Their normal counterparts or the pLTRpoly/ODC in antisense orientation expressing cells retained their epithelioid morphology, remained single cells and formed no foci in soft agar (I: Fig. 1a,d and c,f respectively).

pHβApr1neo vector expressing ODC gene in sense orientation proved to be less potent inducer of transformation. This construct transformed cells with a lower efficiency than the pLTRpoly construct, however, they grew similarly in a criss-cross fashion and were able to induce several foci, but to a lesser extent than the pLTRpoly(S)-cells. pAPr(AS)-cells were totally flat and also did not show any signs of transformation.

1.5 ODC antisense constructs inhibit the v-*src* -induced transformation

When the R2 cells are grown in the restrictive temperature of +39.5°C the *src*-mutant is inactive but becomes activated after downshifting to the permissive temperature of +35°C. The cell morphology changed correspondingly from normal flat (I: Fig. 4d1) to transformed (I: Fig. 4d2). However, when the R2 cells carrying the ODC-antisense construct were grown in permissive temperature the cells did not change their morphology, but remained untransformed instead (I: Fig. 4d3). This result together with that of ODC activity blocked by specific inhibitor DFMO in Rat-1 LA29 cells (also carrying temperature sensitive RSV mutant) preventing transformation (I: Fig. 4a1-3) led us to suggest that ODC might transduce the transforming activity of Src.

1.6 ODC in other transformation models and human cancers

Soon after the publication of (I) Moshier et al. reported similar results, supporting the idea of ODC having a role in cell transformation [502]. Also, ODC induction has subsequently been reported in various rodent cell systems to be critical for neoplastic transformation [65] or to have transforming potential in combination with other oncogenes, like Ras, v-Src and Myc [15, 136, 137, 489]. Importantly, several transgenic mouse models have further demonstrated that ODC activity is sufficient for tumor promotion [503-506], even though systems that fail in transformation have been reported too [507]. Also, the functional role of ODC in prostate tumorigenesis has been linked to malignant transformation of prostatic epithelium first time not only *in vitro* models, but also *in vivo* mouse experiments and patient samples by Shukla-

Dave et al [508]. In addition, various oncogenic stimuli and genetic alterations in ODC have been shown to cause ODC dysregulation, as outlined in Table 5.

Table 5. ODC is dysregulated by a variety of oncogenic stimuli and genetic alterations in the ODC gene

Stimuli	**Reported action**	**Reference**
Oncogenic Ras	ODC expression and activity dramatically induced	[15, 137, 489]
c-Myc	ODC gene is a transcriptional target of c-Myc	[63, 136, 140]
TPA	Increased ODC translation leads to ODC hyperactivity in response to transformation	[509]
Overexpression of translation initiation factor eIF4E	Increased translation initiation of ODC leads to induction of ODC activity	[65, 510]
Genetic alteration		
Alternative splicing of ODC mRNA	ODC protein truncated and stabilized in human hepatocellular carcinoma	[511]
ODC 3'-UTR (bases 1851-2151)	ODC mRNA stability in carcinomas is dependent of the activity of mTORC1	[100, 101]
Point mutation in ODC gene	ODC expression is increased in response to Myc	[512]
SNP in the 1st intron of ODC gene	High ODC activity is a mediator of tumor promotion	[52, 96]
*ODC*1 +316 SNP	A genetic marker for colon cancer risk and prognosis	[513, 514]

2. Signaling in ODC-, c-Ha-ras -oncogene and v-src-transformed cells and transformation-specific convergence points

The proliferation of normal cells is tightly regulated by the growth factors in the cellular environment. In contrast, the escape of transformed cells from this control of growth factors is a hallmark of cancer [281]. Supposing that a transformed cell should display constitutive or inappropriate activation of one or more components in the signaling pathway to gain independence from the upstream growth factors and, asking if transformations induced by different oncogenes would converge to a shared intermediate, we decided to search for common signaling events in various transformation models. By comparing c-Ha-*ras*^{Val12} and v-*src* transformed cells to ODC-induced cell transformation using the well-characterized PDGF-signaling in normal cells as a reference, we wanted to elucidate these signaling events.

Even though the PDGF-signaling events have been relatively well documented in cells transformed by HRAS -oncogene (the most commonly activated oncogene in human cancers), several conflicting results had remained in the literature [515-518]. Similarly, regarding v-*src*, the first and most studied oncogene, discrepant results have been reported of the PDGF-induced signaling in v-src transformation [518, 519]. The ODC-induced signaling events were almost fully uncharacterized. Notably, ODC may be a central player in the signaling of many oncoproteins, as its activity is constitutively elevated in many cells transformed by different chemical carcinogens [30] and oncogenes, like v-*src*, *neu* and *ras* [13, 15, 17, 135]. Further, it has been shown to be a transcriptional target of c-myc and c-fos oncogenes [63, 520], and v-src-induced transformation can be blocked by transcription of ODC antisense RNA or by ODC-specific inhibitor (I) [521], making it a very interesting model to study.

2.1 c-Ha-*ras*^{Val12}- and ODC-transformed cells show reduced protein tyrosine phosphorylation in response to PDGF stimulation

Our analysis of the phosphorylation status of several signaling molecules, like PLCγ-1, GAP, Nck, Syp or Shc, previously indicated to have transforming activity or to be constitutively activated in different cancer cells showed that none of these molecules are, however, characteristic of cells transformed by c-Ha-*ras*^{Val12} or ODC (II: Fig. 2).

Activation and phosphorylation of MAPKs (Erk1/Erk2) by their upstream kinases upon growth factor stimulation plays a key role in the activation of several transcription factors [522]. Constitutively active MEK1 (kinase upstream of MAPKs) is also shown to be able to transform NIH3T3 cells [523] and MAPKs themselves have been found to be activated in cells transformed by several oncogenes, like *ras*, *src*, *raf* and *mos* [220, 523-525]. To our surprise, no constitutive upregulation of Erk1 or Erk2 was found in *ras*-, ODC- or v-*src*-transformed cells, assessed by electrophoretic mobility shift in Western blot or activity increase in kinase assay (II: Fig. 4). The mobility shift and increase in kinase activity were readily seen upon PDGF stimulation of normal cells. We conclude that the MAPK (Erk1/Erk2) pathway is not responsible for the propagation of the proliferative signals and maintenance of the transformation by the ODC-, *ras*- and v-*src*-oncogenes in these cell lines.

2.2 Sos-1 and Raf-1 exhibit a constitutively retarded electrophoretic mobility in all three transformed cells lines

Translocation and concomitant phosphorylation of Sos-1 from the cytoplasm to the receptor complex on the plasma membrane have been shown to be required for Ras activation and cell transformation [526]. Sos-1 converts inactive GDP-bound Ras to active GTP-Ras, which in turn is able to interact and activate serine-theronine kinase Raf-1[527, 528]. Activated Raf-1 shows a reduced mobility in agarose gel [528]. In line with these earlier results and similarly seen in PDGF-stimulated normal NIH3T3, or Rat-1 LA29 cells grown in non-permissive conditions, *ras*-, ODC- or v-*src*-transformed cells presented constitutive and retarded migration of Sos-1 and Raf-1 (II: Fig. 3). Interestingly, we did not find the MAPK activity to correlate with the phosphorylation state of Sos-1 or Raf-1 in *ras*-, ODC- or v-*src*-transformed cells and therefore, the phosphorylating kinase is suggested to be a different from Erk1 and Erk2.

2.3. Downregulation of the PDGF α- and β-receptors in NIH3T3 cells transformed by *ras* and ODC, and v-*src* induced transformation of rat fibroblasts causes a preferential decrease of PDGF β-receptor

Previous results have shown that many oncogenes can modulate the function of growth factor receptors and induce autocrine loops [277]. Cells transformed by *ras*, for example, have been found to become refractory to PDGF stimulation and display loss or suppression of some of the downstream effectors [515, 518, 519] or expression of some growth regulatory genes [15, 516, 529]. Both normal level of PDGF receptors [518, 529, 530] and of suppressed receptor autophosphorylation [530] that would interfere with the binding of signaling molecules to the receptor have been reported.

We found a marked reduction in PDGF α- and β-receptors in *ras*- and ODC-transformed cells in comparison to the normal cells, shown by both protein and mRNA levels and by receptor

binding assay (II: Fig. 6). Since this was in contrast with the previously published data in *ras*-transformed cells, we considered the possibility that the opposite results could be derived from different oncogene expression levels. Indeed, the comparison of the two *ras*-transformed cell lines E2 and E4 showed the decrease of receptor levels being much stronger in E4 cells, expressing much higher *ras*-levels than E2 cells. Similarly, the difference in oncogene expression levels was also seen in phosphorylation of PLCγ and GAP proteins, previously reported with discrepant results (II: Fig. 7). Also, PDGF β-receptor level in Rat-1 LA-29 cells following v-*src* induction, was profoundly decreased correlating with the degree of morphological transformation. We conclude that the oncogene expression levels determine the degree of aberration in signaling and may explain previous conflicting results.

2.4 The *ras*-, ODC- and v-*src*-transformed cells display a constitutive increase in phosphorylation of c-Jun on serines 63 and 73

To follow our idea of finding a constitutively active signaling mediator, we analyzed nuclear fractions of *ras*-, ODC- and v-*src*-transformed cells by Western blotting. Previously, c-Ha-*ras*-induced c-Jun phosphorylation on its transactivation domain has been detected by ^{32}P-orthophosphate labeling [345, 531]. In line with these results, we detected a constitutive increase in phosphorylation of c-Jun on serines 63 and 73 in all three transformation models, using phospho-specific antibodies to c-Jun (II: Fig. 8a,b).

Since we had already excluded the MAP kinases (Erk1/Erk2) and the JAK-STAT pathway (II: Fig. 5) as potential kinases constitutively active in *ras*-, ODC- and v-*src*-transformed cells, the possible activation of c-Jun kinases (JNKs) was examined. The JNK1 and JNK2 are thought to be the principal kinases responsible for c-Jun phosphorylation on its transactivation domain on serines 63 and 73 [344, 345]. Our results in ODC-transformed cells show that JNK1 activity is highly elevated in these cells (II: 8c) and is in line with the previous results that show an elevated JNK activation in cells transiently transformed with the *ras*- or v-*src*-oncogenes [496, 532]. Intriguingly, we did not find a correlation between JNK-activity both in immunocomplex or solid phase kinase assays, and c-Jun phosphorylation on serine 73 in *ras*- and v-*src*-transformed cells (II: Fig. 8c and data not shown, respectively). This suggests that there is still an unknown kinase that is different from JNK1-3 [533], capable of phosphorylating the activation domain of c-Jun in these *ras*- and v-*src*-transformed cells.

3. Overexpression or block of S-AdoMetDC induces cell transformation in murine fibroblasts

It has been shown in numerous studies that polyamines are associated with cell transformation and cancer development. This together with our previous results that showed ODC to have oncogenic potential when overexpressed (I), prompted us to test if AdoMetDC could have a similar role in cell transformation as well.

3.1 AdoMetDC induces tumorigenic transformation in sense and antisense orientations

We cloned a 1.65kb insert of human AdoMetDC cDNA in sense and antisense orientations in pLTRpoly vector, known to induce high level of expression of inserted genes (I). Two types of rodent fibroblast cell lines were stably transfected with these constructs together with a *neo*-selection marker (pSV2*neo*), mouse NIH3T3 and Rat-1cells. Rat-1 cells have been used in many

in vitro transformation studies due to their low incidence of focus formation and poor growth in soft agar [534]. The empty pLTRpoly vector together with a *neo*-selection marker (pSV2*neo*) were also transfected into the parental cells, providing controls accordingly (4N for NIH3T3 and Rat-1-neo for Rat-1).

The overexpression of AdoMetDC in sense orientation in NIH3T3 cells (Amdc-s) resulted in a complete morphological transformation, with elongated morphology, in a growth without contact inhibition, with formation of multiple foci in tissue culture, with disintegrated actin filaments and in an acquired ability to grow in soft agar (III: Fig.1 Ab,e,h). Very surprisingly, the same morphological and growth features were detected in AdoMetDC antisense construct-expressing cells (Amdc-as). To note, culturing Amdc-as cells in the presence of low spermidine concentration (1 µM) was vital to prevent the counterselection of high Amdc-as expressors, as the expression of the AdoMetDC antisense construct blocks the synthesis of spermidine and cell proliferation. However, Amdc-as+spd cells had a little less elongated morphology (III: Fig.1 Ac), but a similar degree of actin filament disintegration (III: Fig.1 Af) and growth in soft agar (III: Fig.1 Ai). Similar induction of transformation was seen in Rat-1 Amdc-s and Rat-1 Amdc-as +spd cells (III: Fig.1 Bb,c). The parental control cells from both cell lines showed similar epithelioid and stringent density-dependent morphology (III: Fig.1 Aa for NIH3T3, Ba for Rat-1). Also, 4N cells presented normal actin filaments and were not able to grow in soft agar (III: Fig.1 Ad,g).

Northern blotting showed the mRNA expression of AdoMetDC cDNA in NIH3T3 transfectants (III: Fig.2A). Amdc-s cells had an average of 30-40-fold higher constitutive activity than the control cells, with spd-addition having only a marginal effect (III: Fig.2 B. The high AdoMetDC activity seemed to correlate with the degree of morphological transformation, but ODC-activity remained on the level comparable to control, spd-addition having no marked effect either. Thus, we could not find any signs of feed-back control by polyamines of their biosynthetic enzymes that is reported earlier [8, 20], at least at this low level of spermidine.

The Amdc-as cells had, as expected, very low levels of AdoMetDC-activity (III: Fig.2 B left), but interestingly, showed about a 12-fold increase in ODC-activity (III: Fig.2 B right), when cultured with spermidine. We assume this to be a compensatory mechanism, related to the role of AdoMetDC as a rate-limiting enzyme for the higher and vital polyamines. By keeping up the ODC activity and thus putrescine production, these cells have better a change to survive. The polyamine content of Amdc-as+spd cells (III: Fig.2 C) was similar to the one in ODC overproducing cells [466]. As a difference, the increase in putrescine was not that high than in ODC-transformed cells, but instead, more putrescine was further converted to spermidine. In Amdc-s cells the polyamine content was very different in comparison to normal or Amdc-as cells, with almost no putrescine, and the balance being almost solely on the end product, spermine (III: Fig.2 C).

The higher putrescine levels in Amdc-as+spd cells are in line with earlier reports connecting the increase in putrescine to cell proliferation and transformation [466, 504, 535]. However, when we consider the situation in AdoMetDC-induced transformation, where putrescine levels were lower than in normal control cells and the overall polyamine balance was clearly towards the end product spermine, the unconditional importance of putrescine in dysregulated growth could be questioned. Also reflecting the opposite patterns of total polyamines in AdoMetDC- or ODC-induced transformation [466] (Fig 27) encourages us to

suggest that it is rather the imbalanced ratio of polyamines than just a single polyamine change, which leads to cell transformation.

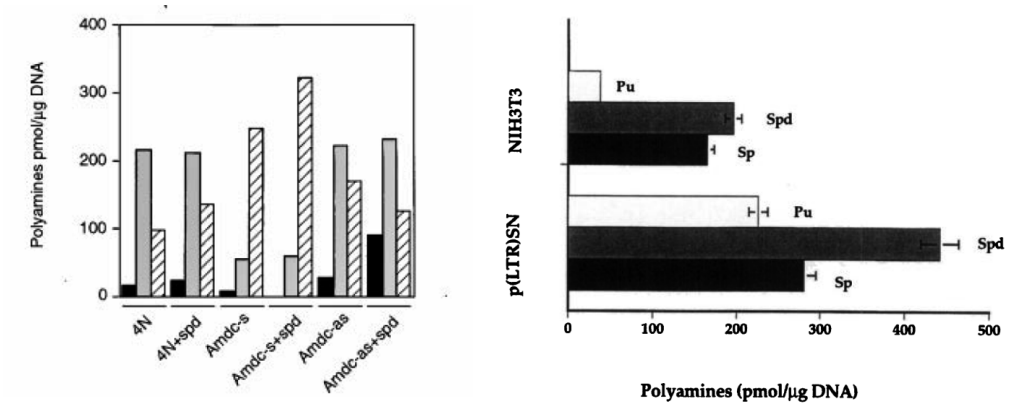

Figure 27. Polyamine levels in AdoMetDC-S and -AS cells (left) (III: Fig2 C) and in ODC-transformed cells (right). On the left black bars = putrescine, gray bars = spermidine, striped bars = spermine for AdoMetDC and on the right white bars = puterscine, dark grey bars = spermidine, black bars = spermine for ODC. Adapted from III and [466].

For the tumorigenicity assays all four cell lines (4N, Amdc-s, Amdc-as and Amdc-as+spd) were injected into the flanks of athymic mice. Only the transformed cell lines were able to induce tumors, but with different potency. Strikingly, the Amdc-s cells were able to induce aggressively growing, highly invasive tumors penetrating rapidly through the muscle and fat tissues into the peritoneal cavity. In a similar assay, the ODC-transformed cells were able to induce large and well-vascularized tumors, but only at the site of inoculation [466].

Of note, Zabala-Letona et al [536] have recently demonstrated a crucial role of dysregulated AdoMetDC activity also in prostate cancer tumorigenesis and validated the same mechanism both in mouse models and in human cancer specimens. These results further support our findings that AdoMetDC has an important function in controlling cell phenotype and tumorigenesis.

3.2 AdoMetDC-induced transformation converges on constitutive phosphorylation of endogenous c-Jun at Ser73

Numerous reports have documented the necessity of Erk1/Erk2 MAPK cascade activation in cellular growth [537] and in several oncogene-induced transformation models MAPK pathway is activated [538, 539]. However, we did not detect constitutive activation of Erk1 and Erk2 in the AdoMetDC-transformed cells, neither by electrophoretic mobility shift in Western blot (III: Fig.3B), nor by in kinase assay (III: Fig.3B). This indicates that the activation of Erk1/Erk2 cannot be responsible for the maintenance of AdoMetDC-induced transformation and therefore is in line with our results with c-Ha-*ras*-, v-*src*-, and ODC-transformed cells (II) and the results of others in other models [540].

Exclusion of constantly activated Erk1/Erk2 signaling pathway as a possible mechanism of AdoMetDC-induced transformation led us to consider other pathways known to be connected to oncogenesis. The parallel pathway of JNK kinases has been shown to be involved not only

in stress-induced signaling, but also in cell cycle control, cell proliferation and transformed growth [343]. The performed kinase assays showed that the endogenous JNK was constitutively activated in NIH3T3 and Rat-1 cells transformed by AdoMetDC overexpression and this activation resulted in the phosphorylation of c-Jun at serine 73 (III: Fig. 4A, B). That the increase in JNK activity was not just a resultant of transformation was excluded by transient transfection of AdoMetDC cDNA into NIH3T3 cells, which showed activation of JNK as well. Interestingly, Amdc-as+spd cells displayed an increase in c-Jun phosphorylation, despite of absent JNK activation (III: Fig. 4B, left). This conundrum is discussed below (see 3.3).

3.3 Dominant-negative mutants of JNK pathway reverse the transformed morphology of AdoMetDC-overexpressing cells

Previous studies indicating the importance of c-Jun and its activating phosphorylation in Ras-transformation [365, 541, 542] together with our results displaying constitutive increase in c-Jun phosphorylation on Ser63 and Ser73 in *ras*-, v-*src*- and ODC-transformed cells (II) stimulated us to investigate the effects of dominant-negative mutants of this pathway in AdoMetDC-induced transformation. Dominant-negative mutants of SEK-1 (immediate upstream activator of JNKs), JNK-1 and TAM-67 (lacking the transactivation domain of c-Jun) were transfected into the 4N and Amdc-s cells. This led to a reversion towards the normal, epithelioid morphology in Amdc-s cells, with an accumulation of multinucleated cells, probably due to the dysregulated cytokinesis (III: Fig. 5A). Expression of TAM67 resulted in the most efficient reversion of the transformed phenotype and into inhibition of soft agar growth (III: Fig. 5Af,g, Ca,b) that was also seen in Amdc-as+spd cells (III: Fig. 5B, Cc,d), in which the DN-SEK1 or DN-JNK1 appeared not to be effective. This might be a consequence of Amdc-as+spd cells showing JNK-independent phosphorylation of c-Jun (III: Fig. 4A left, B left) that could, indeed, be a result of activation of another kinase, different from JNK or Erk1/Erk2. A similar situation, a JNK- and Erk-independent and transformation-specific phosphorylation, has been reported for FosB [543].

Our finding in relation to the constitutive phosphorylation of c-Jun on Ser73 on its transactivation domain in Amdc-s and Amds-as+spd cells and their dominant negative c-Jun (TAM67)-induced morphological reversion towards epithelioid phenotype argues for the importance of transactivation domain of c-Jun in AdoMetDC-induced transformation. To our knowledge this data is the first to indicate that the polyamine biosynthetic enzymes, and thereby polyamines known to be essential for growth, may require the c-Jun transcription factor for eliciting their cellular responses. This is also the first example of a protein whose overexpression or the block of synthesis can lead to transformation. This data emphasizes the fact how strictly the AdoMetDC levels have to be regulated, in order not to disturb its delicate balance.

4. AdoMetDC-induced aggressively growing invasive tumors present chaotic neovascularization

The aggressive growth of AdoMetDC-induced tumors in *nude* mice (III) was remarkable. We were fascinated to study the potential molecular mechanisms behind this haphazard growth model that seemed to result in disorganized angiogenesis, leading to abnormal tumor vasculature and exceptionally high invasive capability.

4.1 Invasive growth *in vitro* and *in vivo*

Amdc-s, ODC-overexpressing (Odc-n), Ras-transformed (Ras E4), human fibrosarcoma (HT-1080), breast carcinoma (MDA-MB-231) and NIH3T3 control cells were cultured in a reconstituted matrix of basement membrane components (3D-Matrigel) and their invasive capacity were compared without any support of other stromal cells. This assay showed that the Amdc-s cells had extensively branched growth pattern, being thus by far the most invasively growing cell line of all cell lines tested (IV: Fig. 2).

This extreme invasive capacity of Amdc-s cells was further manifested by the *in vivo* assay, where they were injected into athymic mice. This induced aggressively and invasively growing tumors (IV: Fig. 1) able to penetrate through the layer of fat, fascia, muscles, and parietal peritoneum and, in uttermost short time, to fill the peritoneal cavity with tumor mass and reach the tissue parenchyma of small intestine, pancreas, liver and spleen. This is a prominent difference from the situation we have seen earlier for ODC overexpressing cells in *nude* mice, in which the transformed cells gave rise to rapidly growing, large and well-vascularized fibrosarcomas at the site of inoculation [466] or other oncogenes (as *ras* or v-*src*) with local invasive properties [544, 545].

4.2 AdoMetDC-transformed cells induce mosaic vasculature with irregular or missing CD31-staining for endothelium

The immunohistochemical analysis of the tumors induced in Amdc-s injected *nude* mice revealed widely varied patterns of endothelial cell marker CD31-staining. Within the same tumor section, we detected mainly vessels that were highly irregular in shape and size, together with some almost regular tube structures. CD31 immunoreactivity showed either clear gaps or lacked completely (IV: Fig. 3 Aa and Ab). Not only the endothelium, but also basement membrane structures depicted by laminin staining, seemed to be aberrant in Amdc-s cells. The deposition of laminin varied from nearly complete to punctuate, or was totally missing (IV: Fig. 3 Ba and Bb). Overall, it seemed that the tumor cells were directly exposed to the lumen of these vessel-like structures. In difference to tumor tissue, the blood vessels in the normal skin tissues on the very same tumor slides, presented complete and homogenously stained layer of CD31-positive endothelial cells and strong staining for laminin (IV: Fig. 3 Ac and Ac).

From these results it was clear that the chaotic neovascularization with irregular formed and sized vessels induced by Amds-s cells was not a result of typical endothelial sprouting, vascular splitting or co-option types of angiogenesis. Instead, two other mechanisms, vasculogenic mimicry [546] or mosaicism [439, 441], were considered as possibilities. Folberg and Maniotis [425] defined vasculogenic mimicry as tumor cells' ability to form fluid-conducting channels rich in laminin and later proposed them to rather dampen than to promote invasive and metastatic phenotype of melanomas [547]. As the vessel-like structures in the tumors induced by Amdc-s cells were lined by tumor cells, but stained not positive for laminin, vasculogenic mimicry was not considered as responsible mechanism for this phenotype.

The lack of CD31-staining, combined with an apparent tumor cell lining of Amdc-s induced tumor vessel-like structures could be an example of mosaicism, even though di Tomaso et al show only focal (and not completely lacking) regions within tumor cells [441]. Our tumor

vessel-like structures lined with tumor cells, but without CD31 positive staining display also mosaic regions, where the CD31-positive staining showed gaps of discontinuity. However, it cannot be concluded that mosaicism would be a sole mechanism to induce angiogenesis in our model.

Interestingly, the publishing of reports describing tumor cells in the vessels of various tumors has continued. So far, tumor cells contributing to the vessel-like structures have been found at least in colon carcinoma [439], neuroblastoma [440] and in glioblastomas [422, 423]. The concept of vasculogenic mimicry has also persisted the initial heavy skepticism towards the idea of microcirculatory channels lined externally by tumor cells, and several cancers with vessel structures resembling vasculogenic mimicry have been reported, e.g. prostate [428], breast [548], gastric [549] and ovarian cancers [550]. The idea of simultaneous or consequent action of possible angiogenic mechanisms was also rehashed recently by a report showing the co-existence of mosaic and mimicry structures in human gastric adenocarcinoma [551].

4.3 Secretion of chemotactic molecules

To test if the Amdc-s cells were able to secrete factors that would stimulate endothelial cell migration, conditioned media (CM) was collected from normal 4N and Amdc-s cells. Their potency to stimulate the migration of bovine endothelial cells in 3D-collagen gel together with CM from control cell lines, the ODC-transformed cells and purified basic fibroblast growth factor as positive and phosphate buffered saline as a negative control was performed. Amdc-s cells were found to secrete even more potent angiogenic factors than the ODC-transformed cells (IV: Fig. 4A), previously found to induce highly vascularized tumors [466].

4.4 Angiogenic switch

4.4.1 VEGF is upregulated in Amdc-s cells

We were interested to see what role VEGF would play in AdoMetDC-induced transformation, as VEGF is known to be a major regulator of abnormal angiogenesis associated with tumor growth [449]. Indeed, Amdc-s cells were found to secrete VEGF in clearly increased amounts (IV: Fig. 4C). By itself, VEGF has been shown to promote only leaky and unstable vessels with fragmented membranes and cooperation with other factors seems to be needed for the maturation of blood vessels [447]. As the levels of secreted VEGF were rather moderate in Amdc-s cells, AdoMetDC-induced angiogenesis is likely to need other pro- and/or anti-angiogenic factors as well. Interestingly, our DNA microarray profilings of the Amdc-s cells and their normal counterparts, revealed up-regulation of several angiogenic proteins, like angiopoietin-like 2 [552] and proliferin [553], possibly contributing to Amdc-s-tumor angiogenesis.

4.4.2 TSP-1 is downregulated in Amdc-s cells and its reintroduction reverses the transformed phenotype and inhibits invasion

As the angiogenic switch is a balance between pro-angiogenic and anti-angiogenic factors, we performed genome-wide DNA microarray analyses to find out, weather other factors than VEGF might be involved in the Amdc-s-induced neovascularization. Affymetrix MOE430 Set -

arrays revealed 20-fold down-regulation of anti-angiogenic factor TSP-1. Also the TSP-1 protein levels were reduced in Amdc-s cells (IV: Fig 5A) and their CM (IV: S Fig 4).

By reintroduction of TSP-1 into Amdc-s cells using a TSP-1-containing expression vector, we wanted to study further the potential role of TSP-1 in AdoMetDC-induced cell proliferation and invasive capacity. TSP-1 expression was confirmed by Western blotting (IV: Fig. 5B). The restoration of TSP-1 in Amdc-s cells inhibited their proliferation (IV: Fig. 5Ca), which was not be due apoptosis (IV: Fig. 5Cb). Importantly, the invasive growth capacity of TSP-1-transfected Amdc-s cells was dramatically reduced in 3D-Matrigel-assay (IV: Fig. 5D).

To our surprise, TSP-2 that shares the same structural domains than TSP-1 and is also a potent inhibitor of angiogenesis [457], was slightly up-regulated (2.9-fold) in Amdc-s cells. In difference to Amdc-s cells, the less aggressive and invasive ODC-transformed cells displayed 7.9-fold down-regulation of TSP-2 in microarray, which is in line with an earlier report [466].

In the very aggressive Amdc-s cells TSP-1 was shown to be one of the most down-regulated genes. VEGF was up-regulated, but only moderately. By comparing this situation to ODC-transformation that shows a similar down-regulation of TSP-1, but no change in VEGF levels [466], we propose that in fibrosarcomas the switch to an angiogenic phenotype and tumor expansion may largely depend on the levels of TSP-1.

4.5 Deregulation of expression of the extracellular matrix proteins

Modulation and degradation of ECM, a multistep process necessary for tumor growth, angiogenesis and invasion, involves many players, like metalloproteinases (MMPs) ([471, 472, 476]. Indeed, genome-wide DNA microarray profiling showed a ~5.3-fold upregulation of MMP-2 in AdoMetDC-transformed cells in comparison to their normal counterparts (IV: Fig. 5A), confirmed also by induction seen in RT-PCR, zymography and as a secreted fully active form in CM prepared from Amdc-s cells (IV: Fig 5B,C,D). However, MMP-9 was not found to be elevated in either of the microarrays (data not shown), RT-PCR nor in zymography (IV: Fig. 5B,C)

As tissue inhibitor of metalloproteinase-2 (TIMP-2) has beside its role as a MMP-2 inhibitor another function together with MT-MMP1 in the activation of MMP-2 [474], we were interested to see its expression as well. Similar to MMP-2, the increased TIMP-2 levels in CM were detected in Amdc-s cells compared to normal 4N cells (IV: Fig. 5D).

4.6 Differential expression of tenascin isoforms

Microarray analyses of Amdc cells revealed also elevated levels of a large extracellular matrix protein tenascin-C that is known to have versatile roles in connection to angiogenesis, malignant transformation and metastasis [484]. The higher-molecular weight isoform of TN-C was shown to be increased in CM of Amdc-s, Odc-n and Ras E4 cells, but not in the control 4N cells (IV: Fig.8A). Intriguingly, TN-C was also richly expressed in the walls of the Amdc-s-induced tumor blood vessels and also, in long ECM-strands lining the tumor cells, similarly to invasive melanomas [554].

As Amdc-s cells showed increased expression of MMP-2 and the large isoform of TN-C, one could speculate that while not being the main triggers of angiogenic switch, they have at least the potency to create a remodeled matrix that would be conducive to tumor cell invasion and, further, to metastasis.

CONCLUDING REMARKS

Polyamines and their biosynthetic enzymes have proved to be indispensable to cells throughout the metazoan kingdom. The strict and very unique control systems have guaranteed the balance in the levels of different polyamines, as any aberration could be fatal to cells. Ornithine decarboxylase (ODC), the first and key enzyme in polyamine biosynthesis, has gained a special focus: it is rapidly reacting to exogenous signals like growth factors and becomes constitutively active in transformation induced by carcinogens, viruses or oncogenes. Our question, whether ODC is just an innocent bystander or even critical for cancerous growth, needed to be answered. Here we show by overexpressing ODC at constant high levels that it is able to induce full morphological transformation, increase cell proliferation and anchorage-independent growth in immortalized rodent fibroblasts. Also, blocking of the ODC activity by synthesizing antisense RNA or using a specific inhibitor of ODC, reverts the v-*src*-induced transformation. These results suggest that ODC should be recognized as a proto-oncogene, acting at the convergence point in the signaling pathways induced by several oncogenes. This makes ODC a potentially good target exploitable in cancer therapy. For example, Myc-driven neuroblastomas and lymphomas are conditions, in which inhibition of ODC as a Myc target may be effective and bypass the difficulty of direct Myc inhibition [140, 555, 556]. Hence, ODC and polyamines continue to be promising targets for anticancer therapy design [3, 7].

By analyzing the PDGF-induced signal transduction pathway in v-*src*, c-Ha-*ras*^{Val12} and ODC-transformed cells we were able to answer our second question about possible common denominators in transformation. The fact that the signaling in all three types of transformation models indeed culminated in phosphorylation of c-Jun Ser73 in its transactivation domain, was very interesting. As the complexity of the signaling pathways has increased and the pathways intersect at multiple points, it has become increasingly difficult to ascribe distinct biological functions with a precision. Already the fact that the phosphorylating kinase responsible for c-Jun activation is not the same in these three transformation models, describes the difficulty of the effort well.

The results of AdoMetDC-overexpression in sense and antisense orientations in rodent fibroblasts were intriguing. To our knowledge, this is the first time, when an overproduction or a block of an activity of a single enzyme is transforming. Also, the AdoMetDC-transformed cells showed a c-Jun phosphorylation on Ser73 and the introduction of DN c-Jun mutant TAM67 reverted the transformed phenotype and abolished the anchorage-independent growth. It is tempting to speculate that the phosphorylation of c-Jun or specific AP-1 components would be an important point of convergence in the transforming action of AdoMetDC and many other oncogenes.

We show also that AdoMetDC-transformation induces highly invasive and aggressive tumors in mice, characterized by chaotic angiogenesis. The induction of neovasculature or vessel-like structures that have, instead of endothelial cell layer, luminal areas with cells only from the tumor or originating from the tumor but further evolved, has far-reaching consequences – it is an open route for metastasis. Many anti-vasculature therapies have been shown to be disappointing and resulted later in relapse of the cancer. This could be due to the induction of hypoxia in tumors, in which the collapsing and thus non-functional vasculature leads to the lack or reduced oxygen in the tissues [557]. If this type of mosaic vessel

structures proves to be common for other aggressively growing cancers, it could serve as a chemotherapeutical strategy to attack the tumor instead of the vasculature.

Our results, showing ODC and AdoMetDC overexpression leading to cellular transformation have been later confirmed by several other *in vitro* and *in vivo* results. It is notable, however, that these and our results on common mediators in transformation cannot be directly extrapolated to transformation of human cells (*in vitro*) due to the vast diversity and extraordinary complexity of human cancers [191, 558].

As a further concern, initially the signaling pathways were described as linear chains, but are now known to be far more complex: separate cascades interplay, the same kinases and phosphatases function in various pathways simultaneously, more substrates and feedback controls within cascades occur branching them into new networks of interactions [25, 559-561]. This makes therapeutic efforts of cancers much more challenging. Thus, further studies to understand and interpret these networks in the cellular milieu continues to be of significant relevance. Evaluation of these signaling networks and the eventual transforming mechanisms remain a great challenge for research in the future.

FIGURE CREDITS

Figure 2
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Figure 7
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